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A Conception of Tumor Autonomy Based on Transplantation Studies: *A Review*

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The definition of a tumor as an autonomous growth has enjoyed persistent popularity in textbooks of pathology. In such definitions the adjective "autonomous" is employed to express the idea of independence with respect to two different particulars. One of these relates to freedom from the laws restraining and co-ordinating normal tissue growth, and the other concerns release from the necessity of a continued stimulus. The results of a long series of transplantation studies suggest that the concept of autonomy has a broader application to the field of cancer than is indicated by these attributes, and accordingly a brief review of the experience is presented.

It was observed early in the work of this laboratory that the transplantability of spontaneous rabbit tumors varied in relation to their duration (7, 19, 21, 23). Early tumors could not be transferred to normal animals, whereas tumors obtained after metastasis were readily transplantable. The occurrence of a large number of spontaneous tumors, particularly of the uterus and breast, made investigation of this relationship possible (4–6, 10, 22, 24). Biopsy specimens obtained at monthly intervals throughout the course of disease were subjected to morphological study and to various types of transplantation. In general, four types of transplantation were attempted: autologous, or transfer back elsewhere in the same individual; homologous, or transfer to an unrelated animal of the same species; transfer to other tumor-bearing animals; and heterologous, or transfer to normal animals of a different species.

Autologous transfer was almost always successful, even in the case of the very earliest tumors. In distinction, homologous transfer was only successful late in the course of the tumor and was followed by rapid decline of the primary host and death from metastasis. The results of heterologous transfer paralleled those of homologous transfer, and all tumors that grew in normal unrelated rabbits also grew when transplanted to guinea pigs.

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The results of transplantation of early tumors to rabbits bearing spontaneous tumors of the same type were in sharp contrast to those obtained in normal unrelated animals, for takes invariably occurred. The growth rate was generally slow at first but increased in tempo, closely paralleling that of the spontaneous tumor of the new host. Further, the ability to grow in normal animals was attained simultaneously by both spontaneous and transplanted tumors.

It is inferred from these experiments that the ability to survive in the environment of a normal animal is a developmental acquisition and not a property of the tumor from its inception. Prior to the attainment of this property, however, the tumor does possess the ability to survive in the primary host or in other animals bearing similar tumors. Thus, at this stage, survival appears to be dependent on the special constitutional status of the spontaneous tumor-bearing animal. The factors concerned in this status are not operative in normal animals, and, accordingly, the tumors cannot be transferred to normal animals. Later in the course of development, the tumor becomes independent of these factors, or autonomous, and will survive in their absence in normal animals.

Such a conception of cancer autonomy differs from that implied by the usual definition. It predicates independence of the factors concerned in the development of a tumor as well as those concerned

in its genesis and thus applies only to the fully evolved cancer and not to growths in the process of development. Such growths are not autonomous in this sense, but on the contrary are dependent—dependent for their continued existence and development on factors peculiar to the tumor-bearing individual.

Studies of mouse and human tumors give added evidence of the developmental nature of the property of autonomy (13). A series of transplantation experiments similar to those described in the rabbit have been carried out during the course of spontaneous mammary tumors in C3H mice. Transfer of early tumors to unrelated mice or to alien species gave rise to no takes, but, after the occurrence of metastasis, both homologous and heterologous transplantation were successful. In sharp contrast to the results obtained in unrelated mice, transfer to other C3H mice frequently resulted in growth. It should be emphasized, however, that in view of the intimate relationship between the highly inbred members of the C3H strain, transfers of this type are not comparable to homologous transfers in rabbits and possess no more significance from the viewpoint of autonomy than an autologous transfer in this species. Further, the majority of C3H mice eventually develop mammary tumors and, therefore, must carry the factors necessary for the genesis and development of the tumors. Transfer from one C3H mouse to another is, accordingly, not a test of tumor autonomy. Conceivably, the continued influence of the factors carried by apparently normal C3H mice may account for the conversion of a dependent to an autonomous mammary tumor as is occasionally observed after long serial passage in this strain. The specific relation of the factors to mammary tumors is indicated by the fact that despite extended serial passage of several dependent methylcholanthrene-induced tumors in this strain, autonomy did not develop. In other respects, transplantation studies involving methylcholanthrene-induced tumors gave results similar to those obtained with mammary tumors—that is, early growths could be transferred to C3H mice but not to other strains or species, and such transfer only became possible after the induced tumor had resided in the primary host for a considerable length of time.

Autologous and homologous transplantation experiments are obviously not permissible in man, and an investigation of human tumors from the viewpoint of autonomy has been limited to heterologous studies (3, 8, 9, 12, 15, 16, 18, 20). Guinea pigs have been used as the recipient host and the anterior chamber of the eye as a transplantation site. The incidence of spontaneous tumors in the guinea pig is extremely low, and the possibility of the factors concerned in the development of a human tumor influencing a transplant in this species is sufficiently remote that growth may be interpreted as evidence that the tumor has attained independence of such factors. In a series of 123 human tumors subjected to heterologous transplantation, 65 grew and 58 failed to grow.

A determination of the developmental relationships of autonomy in human tumors is less direct than in the case of animal tumors where immediate autopsy can be performed and accurate records of the duration of the tumor are available. Some information can be obtained, however, by a comparison of the biological status of the tumor at the time of transfer (dependent or autonomous) with its anatomical status in the patient with reference to metastasis, the ultimate fate of the patient, and the survival period (17). In the series noted above, all the tumors known to have metastasized were found to be autonomous. When only local nodes were known to be involved and organic metastasis could not be demonstrated by clinical or x-ray examination, 39 per cent were autonomous. In contrast, transplantation was successful in only 29 per cent of cases without lymph node involvement or recognizable metastases. The relationship between autonomy and metastasis suggests that autonomy, like metastasizability, is a late stage in tumor development. The fate and survival periods of the patients yield further evidence in this direction. Sixty-one, or 93.8 per cent, of the patients whose tumors proved to be autonomous are now dead, and only 4, or 6.1 per cent, are still living. In contrast, 46, or 79.3 per cent, of the patients whose tumors were dependent at the time of transfer are alive, and only 12, or 20.7 per cent are dead. The interval between transfer and death averaged 5.5 months when the tumor proved to be autonomous and was increased to 34 months when the tumor proved to be dependent. In the group of patients bearing autonomous tumors and still living, a period of 20 months has elapsed since transfer, while living patients with dependent tumors have survived for an average period of 41 months.

In several instances, consecutive biopsies have been obtained at intervals during the course of a human tumor, and the results of transplantation confirm the relationships noted above. The tumors would not grow in normal animals during the greater part of their observed course, and transplantability, or autonomy, was a late development associated with the occurrence of metastasis and a rapidly fatal termination.

It is concluded from these experiments that, with reference to biological properties, cancers of rabbits, of mice, and of man are not sudden transformations in normal cells but, on the contrary, represent the final step in a developmental process. During the greater part of their course, the tumors are dependent in nature, their continued existence being conditioned by factors peculiar to the tumor-bearing individual. Autonomy, or the ability to survive in the absence of such factors, is a late development and is followed by a rapid acceleration in the fatal course of the disease.

Such an interpretation renders information of immediate importance concerning the factors on which the development of a tumor depends. An experimental approach is difficult, few investigations have been pertinent, and little is known. Experimentation with the mammary and uterine tumors of rabbits suggests that in these cases, at least, the factors are endocrinological in nature. Animals bearing such tumors show widespread endocrine changes suggestive of the continued action of estrogenic hormone. Such changes are not found in normal rabbits, and the possibility arose that they might be concerned in the development of the tumor. Accordingly, a group of rabbits was subjected to the long-continued administration of estrogenic hormone in small doses, and, following this treatment, a mammary tumor found to be dependent by transfer to normal animals was transplanted to their eyes. A comparable experiment utilizing a dependent uterine tumor was also carried out. In both cases, the transplanted tumors survived and grew. It seems probable, therefore, that in these special cases the constitutional state incident to the long action of estrogenic hormone supplied the factors essential to the continued existence of the tumors.

It should be emphasized that these are special cases, and there has been no evidence of the operation of estrogenic hormone in the development of tumors of other organs. The effect of castration or of the administration of estrogenic hormone in patients with prostatic tumors is highly suggestive that such tumors may be dependent on androgenic hormone. Further, the results of the treatment of human mammary tumors with estrogen or testosterone may be based on the neutralization of dependent factors.

Unfortunately, cancer has been studied predominantly as a local lesion, and the constitutional status of the tumor-bearing animal has received relatively little attention. The existence of an altered constitution is readily demonstrable in the changed susceptibility to the transfer of heterologous tumors. The Brown-Pearce rabbit tumor grows poorly, if at all, in normal C3H mice, but, when transplanted to C3H mice bearing spontaneous tumors, takes invariably occur, and growth is rapid (14). In like manner, the Rous chicken sarcoma grows on subcutaneous transfer to tumor-bearing C3H mice, but takes have not been obtained in normal C3H mice.[1] The growth of homologous tumors is enhanced, and passage of a transplantable mouse tumor to tumor-bearing mice is also associated with the more rapid occurrence of metastasis. A definitive basis for the differing behavior of the tumor-bearing animal is not known. A pronounced variation in ascorbic acid metabolism has been observed in this laboratory, but experiments undertaken to determine its pertinence are not complete.

It has been found that the factors concerned in tumor development are constitutional in distribution and are not localized at the site of the primary growth (21). Fragments of early dependent uterine tumors have been transferred to various parts of the rabbit's body, and serial biopsies with transplantation studies of the resulting growths have been carried out at intervals throughout the remainder of the animal's life. Autonomy was attained at all sites of autologous transfer, and no significant variations in the time of attainment distinguished the uterus from other regions.

It is of interest in passing to note that in the experiment cited in the previous paragraph a considerable variation in growth rate characterized the behavior of the different autologous transplants when transferred to normal animals. This variation was sometimes extreme. In one case, transplants from the uterus grew to fill the anterior chamber of a normal animal in 3 weeks, whereas fragments obtained from an anterior chamber transplant in the primary host required 3 months to reach a comparable size in normal animals. The absence of a relationship between autonomy and growth rate has also been suggested by other experiments, and a determination of the mitotic index of a tumor has been found to be of no value in assessing its ability to grow in normal animals. In like manner, the morphology of the tumor has not been helpful in this respect. The degree of differentiation or organization appears to bear some relationship to the growth rate, but it is of little pertinence as an indication of autonomy.

It is significant that early embryonic tissues, like cancer, possess the ability to grow in alien species (11). Unlike cancer, however, such tissues lose their autonomy with continued development, and by mid-gestation their transplantation reactions are those of normal adult tissue. A shift from

[1] H. S. N. Greene, to be published.

a state of autonomy to one of dependency has not been observed during the course of the tumors studied in this laboratory, but inasmuch as the attainment of autonomy and metastasis are coincidental occurrences, the interval of time available for such a development is sharply limited. In Earle's tissue culture studies, the period of observation is not restricted to the duration of a tumor-bearing individual's life, and extended investigations are possible. He noted that cells subjected to the action of methylcolanthrene in tissue culture became autonomous, but, after continued culture in the same environment, the ability to grow on animal transfer was lost (1, 2). The inferences contained in these observations invite investigation, but, aside from the possibility that the autonomous state may be reversible, there is special significance in the fact that an attribute of such singular and distinctive nature as autonomy characterizes embryonic tissue as well as cancer. The sharing of this property in addition to a close resemblance in morphological, immunological, and biochemical constitution focuses attention on one outstanding dissimilarity in behavior. In both primary and experimental hosts, embryonic tissue undergoes differentiation, whereas cancer does not. The implied suggestion that the step from embryonic tissue to cancer may be relatively short and concerned with the process of differentiation is not new but remains only partially explored.

Normal adult tissues survive and grow on anterior chamber transfer to other animals of the same species, but always fail to survive heterologous transfer. Apparently, factors essential to their continued existence are common to the species of origin but are not shared by other species. (Inflammatory or foreign body reactions do not always follow transfer of adult tissues to alien species, and the failure of growth would sometimes appear to depend on other than immunological differences.) The more stringent qualifications of tumors in developmental phases are met only by the tumor-bearing animal, but, in an autonomous phase, the independence of antecedent requirements is such that the conditions necessary for survival and growth can be supplied by an alien species. In fact, cancer autonomy transcends zoölogical orders, for the growth requirements of the Rous chicken sarcoma are provided by the rabbit, guinea pig, and mouse (16, 25).

Although the word "autonomy" aptly describes the independent status of cancer, it should be clearly recognized that it serves nothing more than a descriptive purpose and requires translation in terms of biological cell changes as well as a causal explanation. Investigation of the mechanism of autonomy has taken a variety of forms. The possibilities are manifold and may concern either the acquisition or loss of properties. A modification in the former direction, relating simply to the ability of the tumor cell to induce a stromal reaction, might supply the necessary conditions. In the animal body, the tumor cell is completely dependent on its stroma for the essentials of existence, and the success of transfer depends on the ability of the transplanted cells to invoke a stromal reaction in the new host. Accordingly, the dependent nature of early tumors may arise from an incomplete development of this ability, the induction of stroma in a normal animal requiring a greater capacity than is necessitated by the altered responsiveness of the connective tissue of a tumor-bearing animal. Experimentation along such lines has been suggestive, and, in this connection, it should be noted that dependent tumors are transplantable to tumor-bearing animals and, further, that tumor-bearing animals provide a stroma for certain heterologous tumors that fail to survive transfer to normal animals.

On the other hand, there is experimental evidence to indicate that, during its developmental course, the tumor cell loses specificity and, concomitantly, its ability to invoke antibodies. It is conceivable, therefore, that the dependency of an early tumor is no more than a reflection of a destructive antibody reaction following the introduction of its cells into a normal animal and that the autonomy of an older tumor depends on the loss of ability to induce such a reaction.

These and other speculations are the subjects of present study, but whether autonomy represents an enhanced stroma-inducing ability, a loss of specificity, or some other modification in the tumor cell, the fact remains that it is a developmental acquisition and that the primary neoplastic focus and the fully evolved cancer differ with respect to this property. The differentiation of tumors or of tumor stages on the basis of a fundamental biological attribute suggests the existence of comparable differences in metabolic and biochemical constitution. From this point of view, it would appear highly important to characterize tumors with reference to dependency or autonomy in order to insure uniformity of materials in chemical or metabolic investigations. In the field of experimental therapy, a distinction would seem essential, for a variation in the influence of chemical, radioactive, hormonal, or other agents would be in line with the known differences in biological properties. Further, in the general field of cancer research, an interpretation and correlation of re-

sults would be facilitated if the tumors utilized were so defined as to allow a valid comparison with experiments based on different material. In view of the widespread use of mouse tumors and inbred mouse strains in cancer research, it should be re-emphasized that transplantability within a strain is no proof of autonomy.

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Nutritional Factors Affecting Tumor Penetrance in *Drosophila melanogaster**†

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INTRODUCTION

Since 1916 hereditary, pigmented tumors in Drosophila have been studied by Ardashnikov (1), Burdette (3), Hammond (4), Hartung (7–9), Herskowitz (10), Russell (13, 14), Stark (16–19), and Wilson, I. T. (21). Various investigators have repeatedly suggested that crowding may affect the tumor frequency either in the larval or adult stage. It has been shown that flies which have survived densely crowded populations in early life do not survive as long as those reared in optimum environmental conditions (11). In 1942, Russell, in discussing previous work done on crowding effects, suggested that scarcity of food or some other crowding condition may affect tumor incidence in *Drosophila melanogaster*.

In our preliminary experiments on an ebony^{11} tumor stock, with the use of 1- and 8-day egg-laying periods, a significant difference was noted in the tumor penetrance (6). Therefore, this study was undertaken to determine whether crowding itself, or via a nutritionally deficient medium, was instrumental in affecting tumor penetrance. Since the genetic term "penetrance" will be used throughout this report, it seems necessary that it be defined at this point: penetrance is a frequency, measured as the percentage of all animals carrying a gene the effects of which are in any way discernible.

STOCKS AND METHODS

Pigmented tumors were repeatedly observed in our ebony^{11}-scarlet stock of *Drosophila melanogaster*. Animals with tumors were mated to establish a tumor stock. Subsequently, an inbred homozygous line was established by making single paired brother-sister matings for some seventeen generations. This pigmented growth appeared to be dependent upon a single recessive gene in the second chromosome.[1] The pigmented growth was generally found in the abdomen or in the thorax of the imago. When dissected from the former location, the tumor was found floating free in a fluid medium, or surrounded by a mass of yellow-green tissue. It may be that this mass of tissue was the remains of the shed mid-intestinal epithelial cells of a prepupal molt (12). In only three of the thousands of animals inspected was the growth located in the head. Irrespective of location, the tumor varied in color from an intense black to a light brown and appeared as a large mass or as smaller individual particles. The developmental origin and histology of this tumor are being studied at our laboratory. The effect of temperature on its penetrance has been studied and will be reported in the near future.

The basic food elements consisted of the usual agar, molasses, "dead" brewer's yeast, and cornmeal formula (2). Sterilized 1×4-inch vials were filled with food to a depth of about 1 inch. After the food had hardened, 1–2 drops of a suspension of "live" Fleischmann's yeast (about 7.5 gm. in 100 cc. H_2O) was seeded on the food. The vials were then permitted to stand 24 hours to allow for yeast growth before the larvae were introduced. The differentiation between "live" Fleischmann's yeast and "dead" brewer's yeast is based upon the fact that the latter had been subjected to a steam pressure treatment to kill all spores, as a means of preventing any yeast growth. To obtain newly hatched larvae, mass matings of the ebony^{11} tumor stock were made in laying jars containing laying trays of agar and molasses food painted with a suspension of "live" yeast and incubated at 25° ± 0.05° C. The newly hatched larvae were transferred on the tip of a very fine artist's brush to the inner wall of the vial just above the food. By this procedure, all danger of crushing larvae

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[1] Glassman, preliminary data.

by direct insertion into the food was avoided. All animals were reared at 25° ± 0.05° C.

RESULTS

Uniform Age Crowding

Newly hatched larvae in multiples of five were introduced into vials to determine the effect on tumor penetrance of the crowding of larvae of the same age (Table 1).

In concentrations of 5–400 larvae per vial, approximately 85 per cent of the emerged adults per larval concentration were tumorous. Survival values of populations of between 5 and 100 larvae per vial showed a random variation around 74 per cent. This figure declined in the last three concentrations of 200, 300, and 400 larvae per vial to values of 66, 57, and 53 per cent, respectively, with no change in penetrance. From the data in Table 1 it is seen that tumor penetrance was unaffected by the progressive crowding of larvae of uniform age. Yet, with the increasing density in population, the survival value was lowered one-third. Since in simple crowding the penetrance values of preliminary experiments were not obtained, a new approach to the problem was essential. Therefore, an attempt was made to simulate the natural egg-laying conditions.

TABLE 1

UNIFORM AGE CROWDING EFFECTS, 2 TRIALS

Larvae per vial	Larvae tested	Imagoes	Tumorous imagoes	Per cent penetrance	Standard error	Per cent survival
5	200	143	135	94.4	0.0200	71.5
10	590	412	375	91.0	0.0140	69.8
15	585	441	378	85.7	0.0165	75.4
25	500	365	294	80.5	0.0140	73.0
35	700	557	495	88.9	0.0131	79.6
50	1,000	741	649	87.6	0.0119	74.1
100	2,000	1,491	1,307	87.7	0.0084	74.6
200	3,800	2,499	2,102	84.1	0.0072	65.8
300	6,000	3,394	2,299	67.7	0.0080	56.6
400	8,000	4,243	3,735	88.0	0.0047	53.1

Crowding by Simulated Egg Laying

In this procedure, several requirements had to be met: (*a*) The experiment must be equivalent in time to the 8-day laying period in which tumor penetrance had been affected. (*b*) The data (Table 1) demonstrated that 100 larvae were the maximum number that could be used without change in either the penetrance or the survival values; this is the mean number of eggs laid in 24 hours under optimum conditions (5). (*c*) A daily population of 40 larvae per vial would provide a control concentration.

Penetrance.—The environmental conditions, produced by egg laying during a number of days in the same vial, were duplicated by introducing known numbers of newly hatched larvae into the same vial daily for a number of days (Tables 2–4). The data show that, when the concentration of 40 larvae daily for 5 days was attained, the penetrance value of 79.3 per cent fell within the range of random sampling when compared to the penetrance value of 84.1 per cent produced by placing the same number of larvae (200) in vials at one time (Tables 1, 2). When 100 freshly hatched larvae were placed in each vial daily for 4 days, the penetrance value of 66.1 per cent was approximately 22 per cent lower than that resulting from placing the same number (400) in vials at one time (88 per cent penetrance, Tables 1, 3). When 100 freshly hatched larvae were added to each vial daily for 8 days, the penetrance figure, 64.2 per cent (Table 4), did not differ significantly from the 66.1 per cent value (Table 3) produced by placing 100 larvae in vials daily for 4 days. However, a comparison of the daily penetrance values in Tables 3 and 4 demonstrates a marked difference.

TABLE 2

CROWDING BY SIMULATED EGG LAYING: RESULTS OF PLACING 40 LARVAE DAILY FOR 5 DAYS IN EACH OF 20 VIALS

2 Trials (4,000 Larvae); Survival: 55.9 Per Cent

Day	Imagoes	Tumorous imagoes	Penetrance per cent	Standard error
1st	187	161	86.2	0.0252
2d	410	318	77.6	0.0200
3d	312	247	79.1	0.0200
4th	299	221	73.9	0.0252
5th	277	217	78.3	0.0252
6th	190	154	81.1	0.0282
7th	267	220	82.4	0.0220
8th	161	135	83.9	0.0282
9th	132	99	75.0	0.0374
Total	2,235	1,772	Av. 79.3	

In the 8-day trials (Table 4), the penetrance was constant for the first 5 days (78 per cent); consistent day-by-day reduction in penetrance occurred from the sixth through the fifteenth days—the penetrance value on the fourteenth and fifteenth days being 24.2 per cent, less than one-third

of the constant value of the first 5 days' emergence (78 per cent).

Survival.—When either 200 or 400 larvae were inserted into vials over a period of days, emergence was delayed 2–4 days in both cases; and about two-thirds of the surviving imagoes appeared during the first 5 days of the emergence period. There was no marked difference in survival values when

TABLE 3

CROWDING BY SIMULATED EGG LAYING: RESULTS OF PLACING 100 LARVAE DAILY FOR 4 DAYS IN EACH OF 25 VIALS

2 Trials (10,000 larvae); Survival: 59.0 per cent

Day	Imagoes	Tumorous imagoes	Penetrance per cent	Standard error
1st	720	473	65.7	0.0173
2d	1,353	775	57.3	0.0131
3d	878	510	58.3	0.0165
4th	673	486	72.2	0.0173
5th	673	453	67.4	0.0178
6th	567	361	63.7	0.0200
7th	538	415	75.3	0.0173
8th	452	386	85.3	0.0165
9th	49	42	87.7	0.0469
Total	5,903	3,901	Av. 66.1	

TABLE 4

CROWDING BY SIMULATED EGG LAYING: RESULTS OF PLACING 100 "EBONY" LARVAE DAILY FOR 8 DAYS IN EACH OF 20 VIALS

2 Trials (16,000 larvae); Survival: 50.1 per cent

Day	Imagoes	Tumorous imagoes	Penetrance per cent	Standard error
1st	1,049	798	76.1	0.0127
2d	962	774	80.5	0.0127
3d	559	427	76.4	0.0177
4th	702	566	80.6	0.0152
5th	629	495	78.7	0.0162
6th	495	334	72.8	0.0200
7th	569	354	62.2	0.0200
8th	669	397	59.3	0.0190
9th	526	273	51.9	0.0218
10th	427	181	42.4	0.0238
11th	480	206	42.9	0.0295
12th	339	136	40.1	0.0264
13th	358	130	36.3	0.0253
14th	219	54	24.6	0.0291
15th	62	15	24.2	0.0574
Total	8,009	5,140	Av. 64.2	

200 newly hatched larvae were placed in a vial over a period of days or all at one time, or when 400 freshly hatched larvae were similarly handled (Tables 2, 3). Table 4 shows that when 100 newly hatched larvae were introduced each day for 8 days into a vial, only 50 per cent survived. Approximately 49 per cent of the survivors emerged during the first 5 days of the 15-day emergence period, 33 per cent in the second 5 days, and 18 per cent during the last 5 days. Theoretically, it should take only 8 days for all introduced larvae to emerge. Therefore, the emergence time was doubled and extended to 15 days.

From the preceding data on survival and penetrance, the following observations were made: (*a*) The 8-day egg-laying penetrance value was nearly duplicated. (*b*) One hundred larvae inserted daily into vials during 4 or 8 days, as compared to 40 larvae daily, were necessary to evoke a reduction in tumor penetrance and delayed development. The results on the "8-day ebony[11] tumor culture" present more pertinent information. It was noted that concomitant with the delay in emergence there was an orderly decrease in survival rates (Table 4). Therefore, it was inferred that, with increasing numbers of older larvae within a vial, the developmental processes of newly hatched larvae were so impaired as to cause the resultant mortality and penetrance values. Specifically, increasing numbers of older larvae were consuming food at such a high rate that a nutritional deficiency in terms of available food was created which apparently adversely affected both the rate of development and tumor penetrance of newly hatched larvae.

ANALYSIS OF THE AGE OF THE CULTURE IN RELATION TO PENETRANCE, RATE OF DEVELOPMENT, AND SURVIVAL

To determine which groups of offspring over an 8-day laying period were being affected by severe competitive conditions, larvae of a "white-eye gray-body nontumor" stock were introduced alternately with the "scarlet-eye ebony[11] tumor" stock during intervals of the 8-day period. In one trial, 100 freshly hatched larvae of the white stock were placed in each of ten vials on the first 2 days and the last 4 days of the 8-day period. On the intervening third and fourth days, 100 freshly hatched ebony[11] larvae were placed in each of the same ten vials. In a second trial, 100 newly hatched white larvae were placed in each of ten vials daily for the first 4 days. On the final 4 days of 8-day period, 100 freshly hatched larvae of the ebony[11] stock were placed in the same vials daily. In a third trial, 100 freshly hatched larvae of the white stock were placed in each of ten vials daily for 6 days, followed by the daily insertion of 100 newly hatched larvae of the ebony[11] stock on the last 2 days of the 8-day period. In the last trial, on each of the first 7 days of an 8-day culture, 100 freshly hatched larvae of the white stock were placed in each of ten vials followed by the insertion on the eighth day of 100 newly hatched larvae of the ebony[11] stock in each of the same ten vials.

Penetrance.—When 100 newly hatched ebony[11] larvae were introduced into vials on the third and fourth days, on the last 4 days, on the last 2 days,

and on the last day of an 8-day culture, the penetrance values were 85, 50, 47, and 33 per cent, respectively (Tables 5–8). Therefore, it seems that the tumor penetrance of newly hatched ebony[11] larvae placed in vials on the third and fourth days of an 8-day culture was unaffected by the presence of other older larvae already in these vials. But the tumor penetrance of newly hatched ebony[11] larvae placed in vials on the last 4 days, the last 2 days, or on the last day of an 8-day culture was definitely reduced. This reduction in tumor penetrance is in some way due to changes produced in the environment by the presence of larvae previously inserted and maturing on the same food.

TABLE 5

AGE OF THE CULTURE IN RELATION TO PENETRANCE, RATE OF DEVELOPMENT, AND SURVIVAL

100 LARVAE DAILY PER VIAL

2 Days "white"; 2 days "ebony"; 4 days "white"; "ebony" survival: 44.5 per cent

Days	"White-eye" imagoes	"Ebony" imagoes	"Ebony" tumorous imagoes	Penetrance per cent	Standard error
1–3	1,533				
4–9	1,384	890	748	85.3	0.0100
10–15	1,338				
Total	4,255	890	748	Av. 85.3	

TABLE 6

AGE OF THE CULTURE IN RELATION TO PENETRANCE, RATE OF DEVELOPMENT, AND SURVIVAL

100 LARVAE DAILY PER VIAL

4 Days "white"; 4 days "ebony"; "ebony" survival: 46.4 per cent

Days	"White-eye" imagoes	"Ebony" imagoes	"Ebony" tumorous imagoes	Penetrance per cent	Standard error
1–5	2,780				
6–7	185	306	238	77.8	0.0244
8–9	9	508	327	64.4	0.0220
10–11	8	662	261	39.4	0.0200
12–14		381	98	25.7	0.0220
15					
Total	2,982	1,857	924	Av. 49.8	

Rate of development.—Newly hatched ebony[11] larvae introduced into vials on the third and fourth days of an 8-day culture were delayed in their development by 3 ± 1 days; when introduced on the last 4 days, their development was delayed 5 ± 4 days; when introduced on the seventh and eighth days their development was delayed 9 ± 4 days; and when introduced on the eighth day of an 8-day culture their development was delayed 8 ± 4 days (Tables 5–8). These data indicate that delay in development began early in the culture history and increased with time.

Survival.—When freshly hatched ebony[11] tumor larvae were introduced on the third and fourth days of an 8-day culture, their survival value was 45 per cent; when introduced on the last 4 days of the culture, it was 46 per cent; when introduced on the seventh and eighth days, it was 40 per cent; and when introduced on the eighth day, it was 43 per cent (Tables 5–8). These data demonstrate that survival values declined early in the culture history and maintained a constant low state throughout.

Conclusions.—The analysis of the 8-day "all ebony[11] tumor" culture is presented as follows: Tumor penetrance, rate of development, and survival values of a culture history were not directly affected by crowding conditions, but rather by a nutritional deficiency which was related to the increasing numbers of previously inserted larvae maturing on the same food, within a vial. Early in the culture history the rate of development and survival values were impaired (Table 5). Tumor penetrance began to decline in those adults which were placed in the culture as newly hatched larvae on the fifth day. In the presence of food highly depleted by the previous feeding of large numbers of

TABLE 7

AGE OF THE CULTURE IN RELATION TO PENETRANCE, RATE OF DEVELOPMENT, AND SURVIVAL

100 LARVAE DAILY PER VIAL

6 Days "white"; 2 days "ebony"; "ebony" survival: 40.1 per cent

Days	"White-eye" imagoes	"Ebony" imagoes	"Ebony" tumorous imagoes	Penetrance per cent	Standard error
1–10	3,213				
11–12	100	260	132	50.8	0.0309
13–14	52	234	106	45.3	0.0320
15–16	11	185	73	39.4	0.0358
17–18		113	66	58.4	0.0463
19–20		11	4	26.7	0.1330
Total	3,376	803	381	Av. 47.4	

TABLE 8

AGE OF THE CULTURE IN RELATION TO PENETRANCE, RATE OF DEVELOPMENT, AND SURVIVAL

100 LARVAE DAILY PER VIAL

7 Days "white"; 1 day "ebony"; "ebony" survival: 43.1 per cent

Days	"White-eye" imagoes	"Ebony" imagoes	"Ebony" tumorous imagoes	Penetrance per cent	Standard error
1–11	3,316				
12–13	234	63	26	41.3	0.0616
14–15	111	261	87	33.3	0.0292
16–17	3	82	24	29.3	0.0500
18–19	1	25	3	12.0	0.0648
Total	3,665	431	140	Av. 32.6	

larvae, an apparent "floor" penetrance of approximately 33 per cent (Table 8) was noted in newly hatched larvae placed in a vial on the eighth day of a culture. The above data would seem to suggest that delay in development affects tumor formation; but, as will be shown later, development only parallels penetrance values and does not affect tumor formation.

Nutritional Variations

Ardashnikov (1) and Russell (14) found that the appearance of tumors in a Drosophila culture was inversely proportional to the degree of crowding. Sang (15) observed that larvae hatching from eggs within a culture find themselves under different conditions as the culture develops. The changes in food conditions were directly related to the increased number of larvae as well as the numbers of older larvae and to the availability and kind of yeasts within a culture. It has also been shown that certain fractions of yeast and amino and nucleic acids are necessary in the food medium of the developing *Drosophila melanogaster* to insure normal development (20, and 22–30). In the light of these data a preliminary food study was made to determine the effect of nutritional deficiency upon tumor penetrance.

In these last experiments involving a quantitative variation in the basic food formula (see "Stocks and Methods"), 100 eggs per vial of the ebony[11] tumor stock were used in place of larvae. Before the eggs were placed in vials, they were subjected to a water bath followed by an inundation of 95 per cent alcohol to kill any live yeast spores that might have adhered to them on the laying trays. Ten vials per group were used with the following variations in food:

a) Cornmeal-molasses food was used, omitting the "dead" brewer and "live" Fleischmann yeast complements; and 20 mg. per cent of polyvitamin (Polyvitamin Dispersion, Mead, Johnson & Co., Evansville, Ind.) was substituted into the basic food formula (see "Stocks and Methods").

b) Cornmeal-molasses formula was used without the "live" Fleischmann yeast seeding.

c) Cornmeal-molasses formula was used without the "live" Fleischmann yeast seeding and with the addition of 20 mg. per cent of polyvitamin.

d) Cornmeal-molasses formula was used without the "dead" brewer's yeast complement; but the seeded "live" yeast was included.

e) Cornmeal-molasses formula was used, seeded with "live" yeast to which was added 20 mg. per cent of polyvitamin.

In the absence of "live" Fleischmann's yeast, only the combination of "dead" brewer's yeast and polyvitamin complements maintained a relatively high penetrance value of 74.5 per cent (food content *c*, Table 9). A high penetrance figure (82.5 per cent) was demonstrable only when "live" Fleischmann's yeast was utilized in the food medium (food content *d*, Table 9). The addition of polyvitamin to the basic food formula did not change this value significantly (food content *e*, Table 9).

TABLE 9

Effect of Nutritional Variations; 100 "Ebony" Eggs per Vial

Food content	Imagoes	Tumorous imagoes	Vials	Penetrance per cent	Standard error	Critical ratio
a	262	81	10	30.9	0.0282	a & c, 13.11
b	581	196	20	33.7	0.0195	a & d, 13.78
c	638	475	10	74.4	0.0173	b & c, 15.42
d	228	188	10	82.4	0.0252	b & d, 15.41
e	487	412	9	84.6	0.0168	d & e, 0.7000

DISCUSSION

In a series of preliminary experiments, it was noted that there was a significant drop in pigmented tumor penetrance for emerged imagoes as a result of an 8-day laying period, as compared to a 24-hour laying period. From this observation it was tentatively inferred that the size of the population might be one of the critical factors for tumor formation.

Uniform Age, Tumor Penetrance, and the Hypothesis

Before trying to simulate actual laying conditions, the effect of various sizes of population upon tumor penetrance was tested. It would appear that real competitive crowding among ebony[11] tumor larvae of the same age had little effect until concentrations of 200, 300, and 400 larvae per vial were attained (survival values of 65.8, 56.6, and 53.1 per cent, respectively [Table 1]). Owing to the 24-hour yeast growth before entrance of the larvae and the small size of the first and second instars, severe competition for food did not occur until the third larval stage of development. In concentrations of 100 newly hatched larvae per vial this competition had no effect. In concentrations of 200, 300, and 400 larvae per vial, the available

food was not sufficient for consumption needs during the third larval instar. At these concentrations this natural selective process occurred, causing a decline in survival value. But this increased mortality was not accompanied by any change in tumor frequency (Table 1). The fact that the penetrance value returned to 88 per cent in the "400 larvae per vial" concentration apparently discounts the possibility that there was a break in the average tumor frequency at the "300 larvae per vial" concentration. It is believed that this variation is merely an example of random fluctuation.

If a nutritional critical period in development for gene expression of the tumor also occurred in the third larval instar, falling penetrance values would accompany increasing population size. Since this was not the case, it may be that a critical period occurs before the third larval instar. Regardless of whether 200 ebony^{11} larvae were placed in vials at one time or over a 5-day period (Tables 1 and 2), no significant alteration of tumor penetrance or survival values was evident. When the number of larvae inserted daily over a 4-day period was increased to 100, the survival (59.03 per cent [Table 3]) was approximately equal to that value resultant from placing the same number (400) in vials at one time (53.1 per cent [Table 1]). The means by which the nutritional deficiency caused equivalent survival values was different. When 400 larvae were inserted at one time, the competition for food available to third instar larvae occurred between larvae of the same age. Therefore, selection was operative upon the entire population within a common period of developmental time. When daily insertions of larvae were made, the nutritional deficiency became steadily worse. Those larvae inserted on the first day had optimum feeding conditions in terms of food available. Therefore, their opportunity to emerge was greater than subsequent insertions. Coincidentally, the over-all survival rates were the same.

Simulated 8-Day Egg Laying and the Hypothesis

The 8-day "all ebony^{11} tumor" culture lends further support to the nutritional deficiency proposal. Theoretically, it should take 8 days for all inserted first instar larvae to emerge. However, it has been shown that not only was the emergence time doubled, but only 50 per cent of the 16,000 inserted larvae survived (Table 4). The data suggest that, in the face of increasingly severe competition for available food, development of certain groups of larvae was delayed so long that emergence of many was never realized. The penetrance values did not decline until the second 5 days of the 15-day emergence period (Table 4). This indicated that a nutritional deficiency was also affecting the penetrance of the tumor at this time. When, in the morphogenesis of the animal, is tumor development inhibited by the nutritional deficiency and the mortality increased?

Analysis of the ebony^{11}, white 8-day culture data presents explanations to both of these queries (Tables 5–8). With larvae of the "white-eye, gray-body" stock, the onset and termination of ebony^{11} emergence was clearly determined. Ebony^{11} tumor larvae introduced on the third and fourth days of the 8-day culture had a very low survival value (45 per cent [Table 5]), and a delayed emergence period (3 ± 1 days), yet the penetrance value of those third- and fourth-day insertions was unaffected (85 per cent). When ebony^{11} first larval instar insertions were made on the fifth through the eighth days of the 8-day culture, or on the seventh and eighth days, or on the last day of the 8-day culture, the survival values did not change significantly (46, 40, and 43 per cent) from the third- and fourth-day survival value (45 per cent). Therefore, early in the culture history survival dropped sharply and remained at the lower value thereafter. However, penetrance did not decrease in the third and fourth day insertions of ebony^{11} tumor larvae into an 8-day culture (85 per cent [Table 5]) but dropped continuously thereafter (49, 47, and 33 per cent [Tables 6–8]). Moreover, the delay in development increased from 3 ± 1 days to 5 ± 4, 9 ± 4, and 8 ± 4 days.

Since: (*a*) the drop in survival was not associated with the decreased penetrance in the culture history; (*b*) the delay in development began earlier in the culture history than the decrease of tumor penetrance and paralleled it later; and (*c*) as food depletion increased with continued larval insertions, the balance of "food consumption versus food available" became progressively poorer and a nutritional deficiency was created, it is inferred that the nutritional critical period for tumor development occurs before the third larval instar.

Uniform Age versus 8-Day Culture Age and the Hypothesis

As further support to the above proposal, a consideration of the penetrance values produced when first instar ebony^{11} tumor larvae were placed in unoccupied vials as opposed to larval insertions during the culture history is essential. When 200 ebony^{11} larvae were placed in unoccupied vials at one time, the tumor penetrance was 84.1 per cent (Table 1). When 100 first instar ebony^{11} larvae were introduced daily on the third and fourth days (200 larvae) or on the seventh and eighth days

(200 larvae) of an 8-day culture, the tumor penetrance values produced were 85 per cent and 47 per cent, respectively (Tables 5 and 7). When 100 ebony[11] larvae were placed in unoccupied vials, the resultant penetrance value was 85 per cent (Table 1). However, when 100 ebony[11] larvae were placed in vials on the last day of an 8-day culture, the tumor penetrance value produced was 33 per cent (Table 8). These data demonstrate: (*a*) when first instar ebony[11] tumor larvae were inserted into unoccupied vials they were not exposed to a food medium altered by older larval feeding; (*b*) when first instar ebony[11] larvae were inserted early into an 8-day culture, the condition of the food medium created by 2 days of larval feeding was not of such character as to cause a decline in tumor penetrance; (*c*) but when first instar ebony[11] larvae were inserted late in the culture history, a nutritional deficiency had been created which caused the low tumor penetrance values.

This problem of a critical period in tumor development before the third larval instar is now under further investigation. Because of the preceding data and conclusions, it is not believed at present that the effect of a nutritional deficiency on the over-all rate of development modifies or affects the frequency of tumor development. It is altogether possible that other environmental factors modify the rate of development and in so doing affect the frequency of tumor formation. Mrs. Emery (unpublished data from our laboratory) has found that there is a correspondence between the effects of temperature on the rate of development and the penetrance of this tumor.

From the preliminary nutritional study performed (Table 9) it seems as though the presence of "live" Fleischmann's yeast, some of whose biochemical fractions are to be found in the polyvitamin compound used, was necessary to maintain high penetrance values. From these data and the information of Sang (15), Tatum (20), and L. P. Wilson (22–30) it is suggested that the nutritional deficiency discussed above is in reality a yeast deficiency. Specifically, the rate of yeast consumption exceeds the rate of yeast growth, and a food deficiency results. We are now investigating whether the amount of food or certain characteristics of food are necessary for tumor formation.

SUMMARY

1. Crowding of itself as a physical stress does not influence tumor penetrance values in the ebony[11] pigmented tumor stock.

2. A nutritional deficiency created by the presence of increasing numbers of third instar larvae causes a reduction in tumor penetrance in younger larvae in the same culture.

3. It is suggested that this nutritional deficiency is in reality a yeast deficiency which is affecting the development of this ebony[11] tumor stock.

4. It is suggested that a nutritional critical period for tumor occurs before the third larval instar.

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A Method of Transplanting Gastric Mucosa to the Anterior Abdominal Wall of the Rat for the Local Application of Carcinogens*

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INTRODUCTION

The incidence of spontaneous gastric adenocarcinoma in experimental animals is extremely low (1, 2, 8, 10, 13), and it is difficult to produce this neoplasm experimentally with carcinogens (4, 5, 11, 12). Technics for transplanting gastric mucosa to the anterior abdominal wall of cats and dogs for the study of gastric physiology have been described (3, 6, 7). No study has been published, however, on the use of this procedure for gastric cancer. A simple method for exteriorizing a portion of the glandular stomach of the rat onto the anterior abdominal wall for the local application of carcinogens is described, with some observations on the changes in the gastric mucosa caused by this exposure.

METHOD

Piebald male rats of the Royal Victoria Hospital Colony, weighing 150–200 gm., were used for the operations. They were all 2–3 months of age, but were chosen on the basis of weight rather than age, because it was found that the stomachs of rats weighing less than 150 gm. were too small to handle easily, and rats weighing more than 200 gm. had so much fat in the mesentery that the right gastro-epiploic artery could not be seen clearly at the operation. On the day before operation the rats were weighed, and the food was removed from the cages, but water was allowed ad libitum up to the time of the operation.

The instruments, sutures, sponges, and drapes were all sterilized for 15 minutes in an autoclave at 15-lb. pressure just before the operations were started but were not sterilized again during a session of 3 or 4 operations. No sterile gowns, masks, or gloves were used, since a brief hand scrub was found sufficient to prevent peritonitis.

The rat was anesthetized lightly with ether in a glass jar, removed, and tied to the operating board. The ether anesthesia was continued intermittently by the use of a 50-cc. beaker as an ether cone. A layer of cotton was placed in the bottom of this beaker and moistened with ether. The hair was shaved off the left side of the abdomen, tincture of merthiolate was applied, and a small sterile drape sheet was placed over the rat, with the edges tucked under the operating board.

A left rectus muscle-splitting incision, starting just below the costal margin, was extended downward for 3 cm. The stomach was delivered into the incision with a smooth forceps, and the mesentery along the greater curvature was held up to the light. The arcades of the right gastro-epiploic artery were then seen clearly, so that a clear space near the pylorus could be perforated with a fine, pointed forceps (Fig. 1). The tip of one blade of a curved Halstead hemostat was then inserted through this hole, and the blades were closed tightly, thereby crushing together a very narrow strip of the anterior and posterior walls of the stomach. This procedure left the blood supply via the right gastro-epiploic artery intact to the greater curvature, which constituted the flap, and, by crushing the tissue, provided hemostasis on the cut edges. The blades of the curved Halstead hemostat used in this operation were filed down to 1 mm. in width, so that only a small amount of tissue was lost by crushing. When placing the hemostat on the greater curvature, care was always taken to include an adequate segment of glandular tissue without any squamous forestomach, but, at the same time, not to impinge on the pylorus. Four No. 60 black cotton sutures, with the ends left 8 cm. long, were then placed on the greater curvature close to the hemostat to serve as stay sutures in the four corners of the future flap (Fig. 2). A scalpel was used to sever the greater curvature from the stomach, while the stay sutures were held taut so that the arterial supply in the mesentery was not damaged with the scalpel. Then this detached portion of the stomach was laid to

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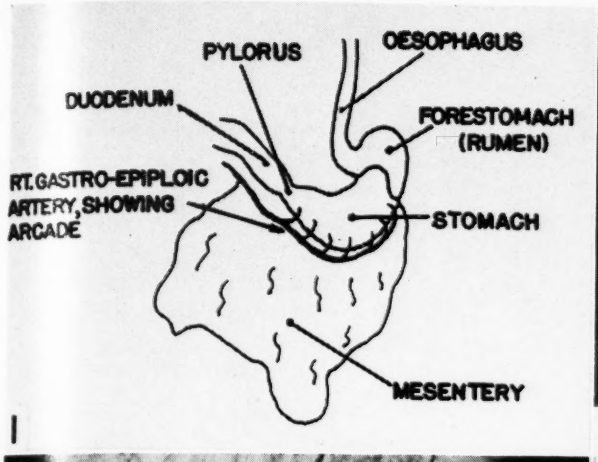
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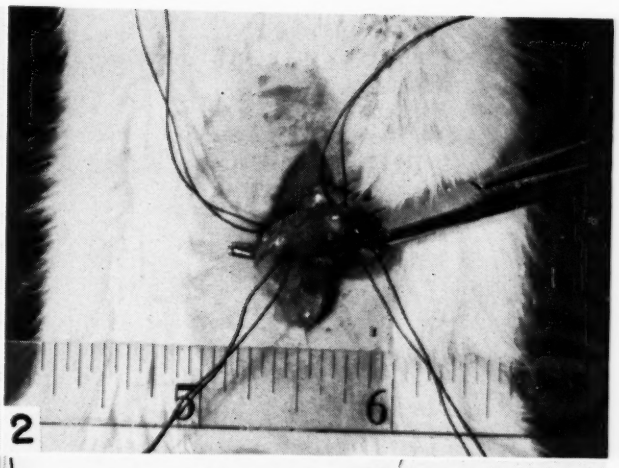
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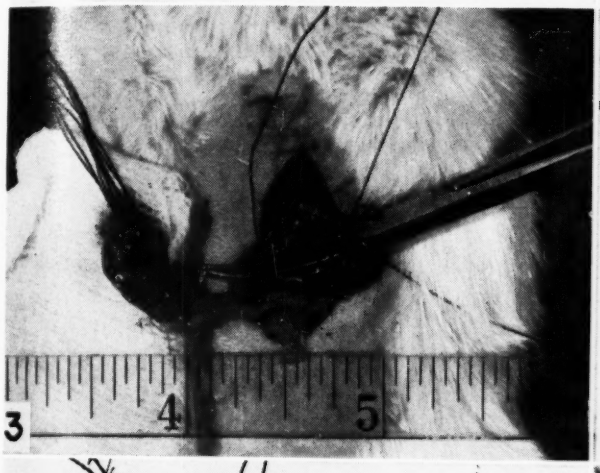
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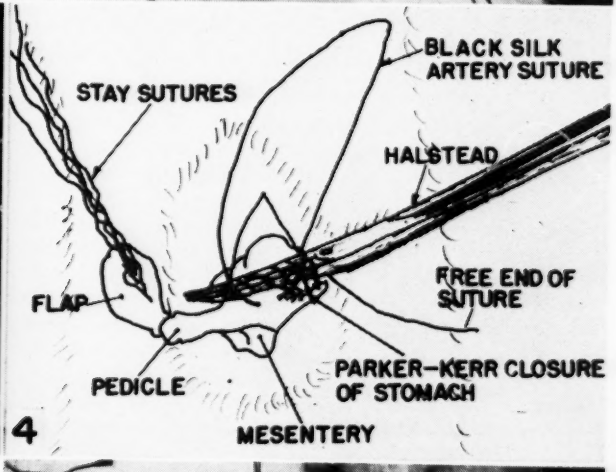
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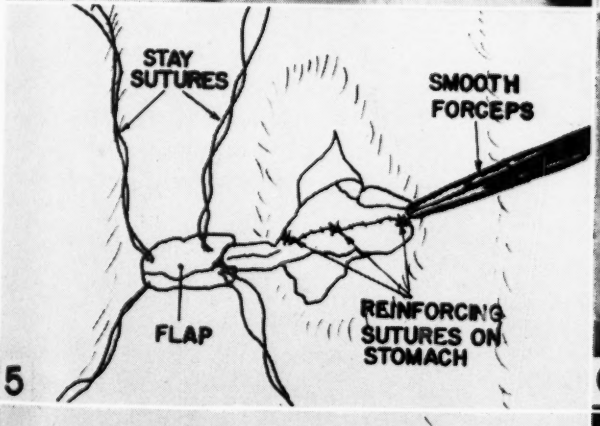
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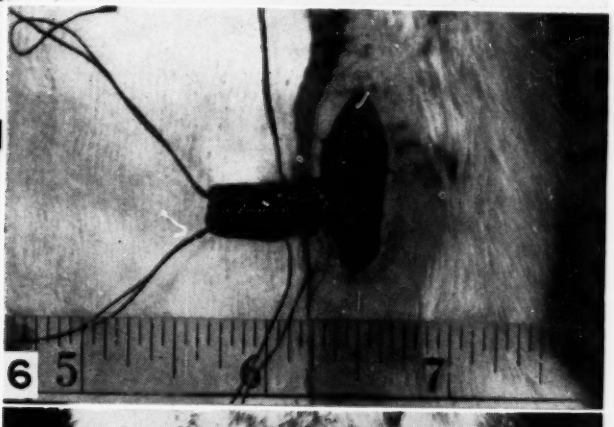
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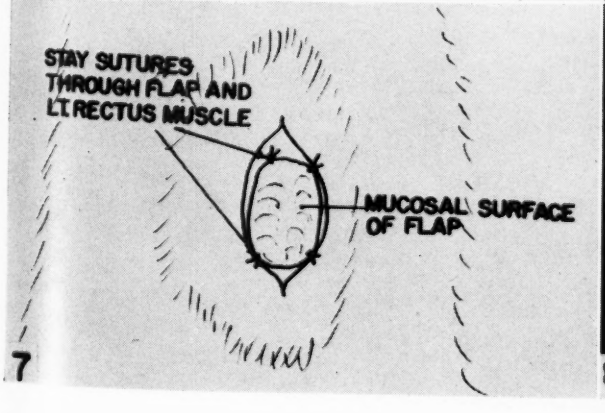
STAY SUTURES THROUGH FLAP AND LT. RECTUS MUSCLE
MUCOSAL SURFACE OF FLAP
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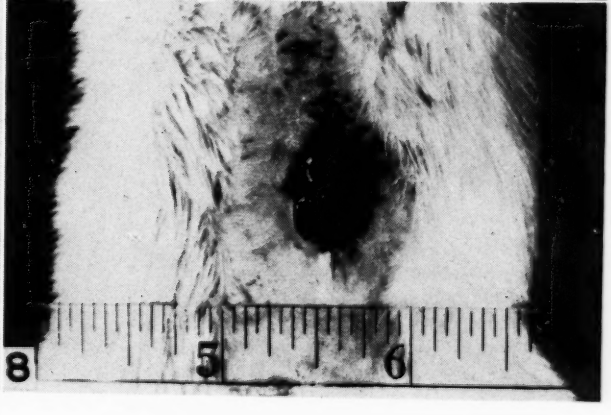
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FIG. 1.—Rat's stomach, showing the right gastro-epiploic artery with the arcades to the glandular stomach.

FIG. 2.—Greater curvature crushed with the Halstead hemostat, showing the four stay sutures.

FIG. 3.—Parker-Kerr closure of the stomach over hemostat.

FIG. 4.—Identification of structures in Figure 3.

FIG. 5.—Stomach closed by Parker-Kerr suture, with reinforcing sutures in place.

FIG. 6.—Left rectus incision closed with interrupted sutures above and below the pedicle to the flap.

FIG. 7.—Flap anchored to left rectus muscle by four stay sutures.

FIG. 8.—Skin edges sutured snugly around the flap.

the right of the incision on the abdominal wall and covered with a sponge moistened in warm saline solution.

A Parker-Kerr method of closure (9) over the hemostat on the cut edge of the stomach was then done with a five-zero black silk artery suture on a curved atraumatic needle (Figs. 3, 4). Several interrupted reinforcing sutures of the same material were placed along the line of closure (Fig. 5), and the stomach was dropped back into the abdominal cavity. The split left rectus muscle was closed with interrupted sutures of No. 60 black cotton above and below the mesentery carrying the blood supply to the flap (Fig. 6).

After the moist gauze was removed from the flap, the stay sutures were separated, and the flap was laid in place, with the mucosa side out, over the closed left rectus muscle. The stay sutures at the four corners of the flap were then used to anchor it to the muscle of the abdominal wall (Fig. 7). The skin edges were pulled over to fit snugly around the flap and were sutured in place with No. 60 black cotton by catching the muscle wall beneath them (Fig. 8). The operating time for this procedure, without an assistant, was usually 30 minutes.

There was very little blood lost during the operation, and the rats were active and drank water within a few minutes. No food was given until 24 hours after the operation.

Skin sutures were removed on the seventh postoperative day, because stitch abscesses developed if the sutures were left in place. By the tenth day, the flaps were well established, and treatment with carcinogens could be started.

RESULTS

More than 300 operations have been done by the use of this technic. Of these animals, 67.5 per cent had good flaps. In 27 per cent the flaps dried up or sloughed off, usually within 10 days after operation. The operative mortality was 5.5 per cent, pyloric obstruction being the major cause of death.

DISCUSSION

After operation, the rats have remained sleek and have gained 3–5 gm. each week, but it has been impossible as yet to tell whether the operation has shortened their life span. A dozen rats with good flaps, from the first series of operations done 15 months ago, are still alive and in good health. Two animals have always been kept in each cage and have never been known to damage each other's flaps.

The skin has always grown closely around the flaps without overgrowing them or ulcerating from the gastric secretion. In 2–3 months after the operation, the flaps have grown thicker and have secreted profusely. Microscopic examination of the flaps has shown that the gastric glands remain, but all the gland cells contain mucin granules when stained with Southgate's mucicarmine stain.

SUMMARY

A simple operating technic for exteriorizing a portion of the glandular stomach of the rat onto the anterior abdominal wall has been described. Good flaps have been obtained in 67.5 per cent of the operations. The rats have remained in good health after operation. Microscopic examination of the flaps has shown that the gastric glands remain, but that all the gland cells contain mucin granules.

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The Effect of Prolonged Administration of 8-Azaguanine on the Growth of Transplanted Adenocarcinoma E 0771*

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In 1949, Kidder and associates reported that the guanine analog 8-azaguanine inhibited the growth of adenocarcinoma E 0771 and of several other tumors in C57 black and other strains of mice (2). The inhibiting effects of 8-azaguanine in several types of adenocarcinoma have since been confirmed (1, 4, 5). The present study deals with the effects of prolonged administration on the growth of adenocarcinoma E 0771.

MATERIALS AND METHODS

Sixty-three mice of C57 black strain obtained from the Roscoe B. Jackson Laboratories in Bar Harbor, Maine, were used. The animals were maintained on a diet of Ralston Purina Fox Chow checkers and water ad libitum. 8-Azaguanine was obtained by courtesy of the Lederle Laboratories Division of the American Cyanamid Company. It was dissolved and administered twice daily by the procedure suggested by Kidder *et al.* (2). Adenocarcinoma E 0771 was transmitted by subcutaneous injection of a sterile suspension of tumor cells into the flank region of the animals.

The effect of prolonged administration of 8-azaguanine was studied in four groups of animals. The seventeen animals of Group 1 and the six animals of Group 2 were 5–6 weeks old at the time of tumor transmission, and the seven animals in Group 3 were 15–16 weeks old. Treatment in these three groups was begun the day the tumor could be palpated as a small nodule, i.e., between 6 and 13 days after transmission. In Group 4, unlike the preceding three, treatment was begun simultaneously with tumor transmission. Group 4 consisted of nine animals 7–8 weeks of age. The daily dose of 8-azaguanine was 1 mg. or about 55 mg/kg in Groups 1 and 4, and 1.4 mg. or about 79 mg/kg in Group 2. Group 3 was started with a dose of 1 mg., which was increased after 3–4 days to 1.4 mg., corresponding to 37 and 52 mg/kg, respectively.

Treatment and observations were continued until all or most of the animals died, except for Group 1. In this group, several animals with very rapidly and very slowly growing tumors were sacrificed.

Control observations on the rate of growth of adenocarcinoma E 0771 were made in nineteen animals 5–6 weeks of age and in nine animals 7–8 weeks of age. The animals were allowed to die spontaneously, except for some which were selected for sacrifice on the basis of extreme rates of tumor growth.

The degree of inhibition by 8-azaguanine was estimated as follows: In the control animals, the rate of growth was found to be different for tumors of different sizes. Since tumor size in the experimental animals varied over a wide range, it was necessary to take the size of the tumor into account. The growth rates for tumors of similar size in the control animals were therefore averaged. The effect of treatment was determined by expressing each daily growth rate as the percentage of the average rate at which tumors of the same size class were found to grow in the control animals. Tumor sizes were obtained daily, as the product of the three principal dimensions measured by caliper. Rates of growth were read from the plot of tumor size on a logarithmic scale against time on an arithmetic scale.

RESULTS

Rate of Tumor Growth in Untreated Animals

The size of the tumors came very close to the figures shown by Kidder *et al.* (2). There was only moderate variation in the rate of growth among different animals. With the increasing size of the tumor, the rate of its growth increased slightly at first; but when the size of the tumor increased to beyond 1,000 c.mm., the rate gradually decreased. The average rate rose from a 35 per cent increase per day for small sizes to 48 per cent for the size class 800–1,000 c.mm., then slowly declined to

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only 15 per cent per day for the class 8,000–15,000 c.mm. (Chart 1).

DECREASE IN GROWTH RATE PRODUCED BY 8-AZAGUANINE

Effect in the first 2 weeks.—Charts 2–4 show the influence of 8-azaguanine on Groups 1–3. During

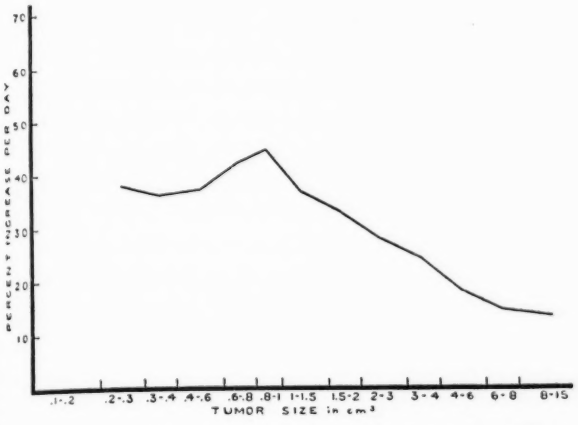

CHART 1 (control animals).—Per cent daily increase in size for tumors of different sizes. The rates shown represent the average of all control animals.

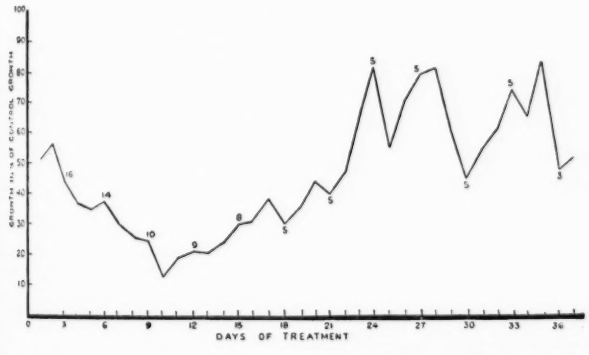

CHART 2 (experimental group 1).—Daily growth rates in per cent of the average rates shown by tumors of the same size in control animals. Numbers on curve represent number of tumor-bearing mice surviving at that time.

the first 2 weeks of treatment, the results were similar in the three groups in which the timing of treatment was the same. Retardation began with the first day after treatment; the inhibition grew more pronounced from day to day and reached a maximum in the second week. The size of the tumors in Group 1 was similar to that reported by Kidder, who employed animals of the same age and a similar dosage. On the tenth day, the rate of growth in these animals was only 13 per cent of that of the controls.

In Group 2, in which animals of the same age as those of Group 1 received a 40 per cent higher dose, the same degree of inhibition as in Group 1 was obtained: the growth rate on the seventh day was 29 per cent and on the eighth day 23 per cent of control growth, as compared to 30 per cent and 26 per cent in Group 1 for the same 2 days. The animals of this group died between 10 and 23 days after the beginning of treatment (Table 1); the animals with the lowest rates of tumor growth were the first to die.

Group 3 contained older animals, and a very low dosage was used initially, which was increased to that given Group 1. The inhibitory effect was

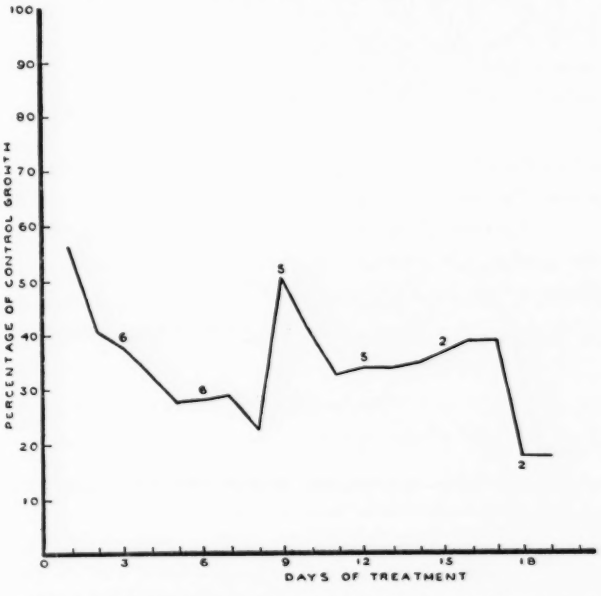

CHART 3 (experimental group 2).—Daily growth rates in per cent of the average rates shown by tumors of the same size in control animals.

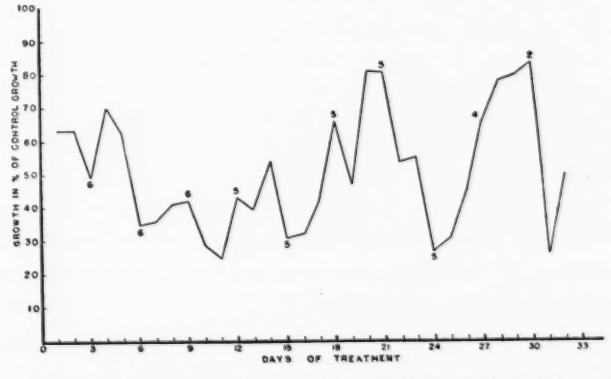

CHART 4 (experimental group 3).—Daily growth rates in per cent of the average rates shown by tumors of the same size in control animals.

very slight at first, and, even after the dosage was increased, it was not so pronounced as that in Group 1. However, it followed the same time course. Maximal inhibition occurred on the eleventh day.

In Group 4, in which treatment was begun on

the day of tumor transmission, not a single tumor developed during the first 2 weeks of treatment.

Effects of prolonged administration.—8-Azaguanine decreased in effectiveness after the end of the second week. A progressive increase in rate of growth followed the maximal slowing seen on the 10th and 11th day. By the 24th day in Group 1 and the 21st day in Group 3, the tumors grew almost as rapidly as in untreated animals—i.e., at 82 per cent and 81 per cent of the control rate.

In Group 4, the first tumors were visible on the 18th day. By the end of the fourth week, the percentage of "takes" was the same as that seen in the other groups. The rates of growth of these tumors were as high as those measured in untreated animals.

Only in the two animals of Group 2 which survived beyond 15 days of treatment did 8-azaguanine retain some of its effectiveness in the third week. Rates of growth between 30 and 40 per cent of the control values were observed until shortly before the death of these animals.

The rapid growth of the tumors seen at the end of the third and beginning of the fourth week in Groups 1, 3, and 4 was followed by fluctuating rates which were of a similar pattern in all three groups. The growth rate in Group 1, after renewed depression, returned to 82 per cent of the control rate on the 28th day and again to 84 per cent on the 35th day. In Group 3, after the peak of 81 per cent on the 20th and 21st days, a second peak of 78–80–84 per cent was reached on days 28–30; in Group 4 a second peak of 76–78 per cent was noted on the 30th and 31st days.

Survival Times of Tumor-bearing Animals with and without 8-Azaguanine

In nine untreated animals allowed to die spontaneously, there was one survivor 52 days after the tumor could first be palpated, and an average period of 18 days between the time the tumor reached visible size and the time of death for the remaining eight animals. The tumor size at death averaged 9.3 c.cm.

In the high-dosage Group 2, the animals survived an average of 16 days of visible tumor growth and of 8-azaguanine administration. However, the average tumor size at death (3.6 c.cm.) was only a third of the control value.

In the low-dosage Group 3, the animals survived 27 days of treatment and visible tumor growth. In the low-dosage Group 4, in which treatment preceded the period of visible growth, they survived an average of 41 days of 8-azaguanine treatment and 18 days of visible tumor growth. In these two groups, the average size of the tumors at death (7.7 and 6.7 c.cm.) was not very much smaller than in the control group.

TABLE 1

SURVIVAL OF TUMOR-BEARING ANIMALS, WITH AND WITHOUT 8-AZAGUANINE

Group	No. animals	Size of tumor at death (c.cm.) Range	Av.	Survival during tumor growth (days)* Range	Av.	Survival during treatment (days) Range	Av.
Control	9	4.3–24.0	9.3	14–52	18		
2	6	1.5– 8.0	3.6	10–23	16	10–23	16
3	7	0.8–15.6	7.7	10–34	27	10–34	27
4	9	0.7–11.4	6.7	4–32	18	33–57	41

* These columns represent the number of days from the appearance of a palpable nodule until the death of the animal.

DISCUSSION

Growth of Adenocarcinoma E 0771 in Control Animals

Our results are in good agreement with those reported by other authors. Gellhorn and collaborators (1) report quadrupling of tumor size in 5 days, which corresponds well with the growth rates observed in our smaller tumors. The tumor sizes reported by Kidder *et al.* (2) are slightly larger than those seen in our experiment, probably because more viable tumor cells are transmitted by the trocar method of transplantation than by the injection of a cell suspension.

The acceleration of growth rate from 35 to 48 per cent increase per day up to the size of 1,000 c.mm. is probably an artifact. The inclusion of two layers of skin with each tumor dimension as measured causes the growth rate to appear lower than it is with small tumors, but this error decreases progressively as the tumor increases in size. The progressively lower rates of growth of larger tumors might correspond to the progressively decreasing fraction of the total tumor mass which proliferates while the remainder undergoes cavity formation, necrosis, and peripheral ulceration.

Growth of Adenocarcinoma E 0771 in the Treated Animals

For the first 2 weeks of treatment, our results are in good agreement with the observations of

Kidder *et al.* (2) and of Gellhorn *et al.* (1). The effect of prolonged treatment with 8-azaguanine has not been reported previously for the E 0771 tumor.

For tumor 755 in C57 mice, Gellhorn and associates report continued inhibition of growth for periods up to 3 weeks, if treatment was begun the day after tumor transmission. These results appear to be in good agreement with those of Group 4 of the present investigation, if allowance is made for the differences caused by the different methods of tumor transmission. When tumor fragments are transmitted by the trocar method, the size of the fragment can be measured soon after inoculation. Inhibition of development by 8-azaguanine will be observed as unchanging size of the fragment. When tumors are transmitted by injection of a suspension of tumor cells, a failure to "take" will be observed for as long as the inhibition persists.

On the other hand, Gellhorn *et al.* report that in one group of five animals in which treatment was begun 10 days after transplantation and continued for 27 days there was "continued slow growth of the tumor without alteration in the growth curve." Treatment in this group was begun only a few days earlier than in our Groups 1 and 3. Gellhorn's observation of continued effectiveness of 8-azaguanine treatment for nearly 4 weeks is thus in disagreement with our findings for these two groups.

A study of the rate of incisor eruption in C57 black mice given similar doses of 8-azaguanine reveals a striking similarity between the effects on the growth rates of continuously growing teeth and those of tumors (3). Eruption of the incisors was depressed for the first 10–20 days. There was in nearly every instance a return to normal rates of eruption in the third and fourth week. Subsequently, the same peculiar periodic fluctuation in the growth rate of the incisors was observed that was noted for the tumors.

SUMMARY

8-Azaguanine was administered to 39 mice of C57 black strain bearing adenocarcinoma E 0771. The day-to-day degree of inhibition of tumor growth was measured by comparison with growth rates of the same tumor determined in control animals. Treatment was continued until all or most of the animals died.

1. When treatment was begun at the time the tumor had reached visible size, the rates of tumor growth progressively decreased during the first 10 days of treatment. With further treatment, a slow return to near control rates of growth took place during the fourth week.

2. When treatment was begun on the day of tumor transmission, tumors failed to develop for about 18 days. Subsequently, tumors showing nearly normal rates of growth were observed.

3. The effects on tumor growth described resemble the effects of 8-azaguanine on the growth rates of continuously growing teeth.

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Choline Oxidase Activity in Rat Liver during 3′-Methyl-4-Dimethylaminoazobenzene Carcinogenesis

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The oxidation of choline to betaine is necessary for the utilization of a choline methyl group in the production of methionine *in vitro* (5, 11). Since the methionine content of the proteins in rat liver tumors induced by azo dyes is very much lower than in liver (12), the effect of a carcinogenic diet containing 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) on the choline oxidase activity of liver before and during development of a tumor has been investigated. The incorporation of 3′-Me-DAB in the diet has been found to produce an early and pronounced inhibition of the liver choline oxidase.

EXPERIMENTAL

Animals.—Male rats of the Wistar strain, which had been maintained on a stock diet of Purina Laboratory Chow until the weight was about 150 gm., were placed on the semi-synthetic diet of Giese, Miller, and Baumann (7) in which 0.06 per cent of the liver carcinogen, 3′-Me-DAB, was incorporated. Control rats of the same weight were fed the basal diet without the carcinogen. The rats were allowed food and water ad libitum and were given 1 drop of halibut liver oil per week. Animals were sacrificed from time to time by decapitation, and the blood was permitted to drain. The typical changes described by Miller *et al.* (10) were noted in the livers from the rats on the carcinogenic diet.

Enzyme preparation.—Liver or tumor was excised from the animal immediately after decapitation and cooled to 5° C. Samples of tissue then were blotted dry, weighed, and homogenized in the presence of an equal part of 0.2 M phosphate buffer, at pH 7.9, in a Potter-Elvehjem glass homogenizer which was cooled in an ice-water bath. The homogenate was dialyzed in cellophane tubing for 21 hours at 5° C. against 2 changes of 100 parts of distilled water, in order to remove naturally occurring oxidizable substrates. The freshly dialyzed homogenate was used; however, there was but little loss in choline oxidase activity during 48 hours' storage at 5° C.

Choline oxidase activity determination.—Oxygen uptake was measured at 37° C. in air in the Warburg manometric apparatus. Each flask contained dialyzed homogenate equivalent to 0.25 gm. of tissue and 0.5 ml. of 0.2 M phosphate buffer of the desired pH in a total liquid volume of 2 ml. For determination of the rate of oxygen uptake, 4 mg. of choline chloride per flask was used as substrate, and the pH of the buffer used was 7.9. The rate was corrected for the oxygen uptake of a similar mixture in the absence of choline chloride. Activity was calculated from the initial linear portion of the rate curve and expressed as c.mm. per hour per gram of fresh tissue.

For determining the maximum oxygen uptake, 1 mg. of choline chloride was used as substrate, and oxidation was allowed to proceed until it was certain that the oxidation of choline had stopped. The initial pH was measured in mixtures duplicate to those in the manometer flasks.

Determination of end-products of choline oxidation.—The protein in an oxidation mixture in a Warburg flask was precipitated by the addition of 3 ml. of 2 per cent sulfosalicylic acid. The protein-free supernatant fluid obtained by centrifugation was used for betaine aldehyde and choline determinations. The betaine aldehyde was determined by the bisulfite method of Clift and Cook (4), which Bernheim and Bernheim (1) had shown could be used for this aldehyde. The choline was estimated by the periodide method (2, 9), which depends upon observation, under the microscope, of characteristic crystals of choline periodide formed in serially diluted samples. The presence of betaine aldehyde and betaine in considerable amounts in proportion to choline noticeably retarded the formation of the choline periodide crystals, even though these products themselves do not form crystals with the periodide reagent (3, 9). The betaine produced was calculated from the oxygen uptake in excess of that required for the amount of betaine aldehyde found.

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RESULTS

The rate of choline oxidation in liver homogenates from rats on the diet containing 3′-Me-DAB was found (Table 1) to be only about 25 per cent of that in liver homogenates from normal and control rats. This markedly lower activity was apparent even in the first animal which had been on the 3′-Me-DAB diet for only 14 days, and was almost as great as it was after more prolonged feeding. The oxidase activity in homogenates of the liver tumor tissue, which had been separated as cleanly as possible from the adjacent liver tissue, was less than 5 per cent of that in homogenates of normal and control liver and less than 20 per cent of that in homogenates of the liver of 3′-Me-DAB rats, an observation in close agreement with the findings of Kensler and Langemann (8) and Viollier (13). Since the activity in tumor tissue was so slight, it may have been due merely to contamination from a small amount of liver tissue.

TABLE 1

CHOLINE OXIDASE ACTIVITY IN LIVER AND TUMOR HOMOGENATES AT PH 7.8

Enzyme source	Rat no.	Time on diet (days)	Weight of rat (gm.)	Rate of O_2 uptake (with 4 mg. choline chloride) (c mm/hr/gm tissue)	Maximum O_2 uptake (with 1 mg. choline chloride) (c mm.)*
Normal liver	N-1		150	1,440	
	N-2		150	1,392	144
	N-3		123	1,920	145
	N-4		210		154
	N-5		200	1,728	158
	N-6		258	1,775	143
Control liver	C-1	36	227	1,440	
	C-2	112	304	1,440	144
	C-3	167	324	1,632	148
3′-Me-DAB liver	M-1†	14	131	480	
	M-2†	21	176	576	92
	M-3‡	42	150	240	69
	M-4‡	62	126	336	105
	M-5‡	104	191	288	106
	M-8§	147	167	240	77
3′-Me-DAB tumor	M-6	127	162	72	33
	M-7	131	169	70	58
	M-8	147	167	6	5

* Theoretical O_2-uptake for 2 atoms per molecule of choline chloride = 160 c.mm.
† Liver appeared normal.
‡ Liver cirrhotic.
§ Tumor masses present in liver.

The low choline oxidase activity in the 3′-Me-DAB liver was reflected not only in the rate of oxygen uptake but also in the total amount of oxygen taken up (Table 1). The maximum oxygen uptake obtainable at pH 7 8 with 3′-Me-DAB liver was close to 1 atom per molecule of choline, while with normal or control liver the oxygen uptake was approximately 2 atoms. The possibility was suggested, therefore, that the enzyme responsible for the uptake of the second oxygen atom might be lacking in 3′-Me-DAB liver.

That liver choline oxidase consisted of two enzymes had been shown by Bernheim and Bernheim (1): the first enzyme catalyzed the oxidation of choline to betaine aldehyde with the uptake of 1 oxygen atom; the second enzyme catalyzed the oxidation of betaine aldehyde to betaine with the uptake of a second oxygen atom. Since 2 atoms of oxygen were taken up at pH 7.8 but only about 1 atom at pH 6.7, they concluded that at pH 6.7 the oxidation of betaine aldehyde was almost completely inhibited. In normal liver homogenate at pH 6.7, however, it has now been found (Table 2) that an oxygen uptake of approximately 1 atom per molecule of choline was not evidence that the choline was oxidized only to aldehyde, because the oxidation had stopped with an appreciable amount of choline remaining unoxidized. Therefore, the oxygen uptake was considerably more than 1 atom per molecule of the choline which was oxidized. Part of the oxidized choline was found as aldehyde, but the oxygen uptake in excess of that required for the aldehyde found indicated betaine formation also. The calculated amount of betaine, together with the betaine aldehyde and unoxidized choline found, accounted for 86–96 per cent of the choline added.

With 3′-Me-DAB livers Nos. 5 and 8 at pH 7.8,

the oxidation picture (Table 3) was similar to that with normal liver at pH 6.7; part of the original choline had been oxidized only as far as betaine aldehyde, but some choline had been oxidized through aldehyde to betaine. A much smaller proportion of betaine was found with No. 8 liver, which was in an advanced state of tumor growth, than with No. 5 liver, which had not yet developed tumor masses. In homogenates of three liver tumors at pH 7.8 there was little or no oxidation of choline beyond the betaine aldehyde stage. The weak oxidase activity of liver tumor and of No. 8 liver was also shown by the cessation of oxidation while a considerable amount of unoxidized choline was still present. With control liver, as well as with normal liver, no aldehyde was found at pH 7.8, no choline was left unoxidized, and the oxygen uptake indicated that choline was oxidized almost completely to betaine.

At pH 7.2 (Table 3) the same qualitative difference between the end-products of choline oxidation with 3′-Me-DAB liver and with normal or control liver was observed as at pH 7.8; the extent of oxidation was, however, slightly less at pH 7.2 than at pH 7.8. At pH 6.7, in a 3′-Me-DAB liver and a liver tumor homogenate, in both of which there was considerable oxidation of choline to betaine aldehyde at the higher pH's, there was little or no oxidation of choline at all; in control liver, as well as in normal liver, there was considerable oxidation with the formation of both betaine aldehyde and betaine.

DISCUSSION

The impairment in the ability of homogenates of 3′-Me-DAB liver and liver tumor to oxidize choline is expressed by the lower rate of oxygen uptake, the smaller total oxygen uptake, the preponderance of betaine aldehyde over betaine as end-products, and the larger amount of choline left unoxidized. An explanation for the cessation of oxidation before all the choline has been oxidized and for the presence of both betaine aldehyde and betaine as end-products may perhaps be found in a greater instability of the enzymes of the choline oxidase system in rats after feeding of 3′-Me-DAB. Eadie and Bernheim (6) showed that the enzyme which oxidizes choline to aldehyde is

TABLE 2

EXTENT OF OXIDATION OF CHOLINE IN PRESENCE OF NORMAL LIVER HOMOGENATE AT PH 6.7

Choline chloride added (mg.)	O_2 uptake: Maximum found (c. mm.)	O_2 uptake: Theory (1 atom) (c. mm.)	Aldehyde found (mg.)*	Betaine calculated (mg.)*	Total choline oxidized (calculated) (mg.)*	Choline found unoxidized (mg.)*	Total choline accounted for (mg.)*	Total choline accounted for (per cent)
1	92	80	0.37	0.39	0.76	0.13	0.89	89
2	177	160	0.95	0.63	1.58	0.14	1.72	86
4	324	320	2.53	0.76	3.29	0.54	3.83	96

* Expressed as choline chloride equivalent.

TABLE 3

EXTENT OF OXIDATION OF CHOLINE IN PRESENCE OF LIVER AND TUMOR HOMOGENATES

Enzyme source	Rat no.	pH (initial)	O_2 uptake (maximum found) (c. mm.)	Aldehyde found (mg.)*	Betaine calculated (mg.)*	Choline found unoxidized (mg.)*	Total choline accounted for (per cent)
Normal liver	N-6	7.8	143	0	0.89	0	89
Control liver	C-2	7.8	144	0	0.90	0	90
" "	C-3	7.8	148	0	0.93	0	93
3′-Me-DAB liver	M-5	7.8	106	0.52	0.40	0	92
" "	M-8	7.8	77	0.58	0.19	0.24	101
3′-Me-DAB tumor	M-6	7.8	33	0.38	0.02	0.54	94
" "	M-7	7.8	58	0.60	0.06	0.31	97
" "	M-8	7.8	5	0.10	0	0.89	99
Normal liver	N-6	7.2	143	0	0.89	0	89
Control liver	C-3	7.2	144	0	0.90	0	90
3′-Me-DAB liver	M-8	7.2	60	0.56	0.09	0.35	100
3′-Me-DAB tumor	M-7	7.2	45	0.54	0	0.41	95
Normal liver	N-5	6.7	92	0.37	0.39	0.13	89
" "	N-6	6.7	70	0.55	0.16	0.22	93
Control liver	C-2	6.7	74	0.46	0.23	0.15	84
" "	C-3	6.7	52	0.54	0.06	0.35	95
3′-Me-DAB liver	M-8	6.7	0	0	0	1.00	100
3′-Me-DAB tumor	M-7	6.7	9	0.11	0	0.78	89

* Expressed as choline chloride equivalent: 1 mg. choline chloride added initially.

a very unstable enzyme, that the stability is greater at pH 7.8 than at pH 6.7, and that the enzyme is more stable in the presence of substrate than in its absence. They suggested that inactivation may result from a combination of the enzyme with some substance present in liver. If this is the case, a greater instability of the enzyme in 3′-Me-DAB liver than in normal liver might be due to the smaller amount of enzyme when the amount of unknown substance remained the same, or to the presence of some substance in excess in 3′-Me-DAB liver.

Since neither choline nor betaine aldehyde, but only the completely oxidized product, betaine, can serve as a methyl donor for transmethylation to methionine (5), the present experiments indicate that there may be a block in the transmethylation system after the feeding of 3′-Me-DAB. The almost complete inability of tumor homogenate to oxidize choline to betaine under the most favorable conditions of pH, 7.8, may explain the low methionine content of tumor protein reported by Schweigert *et al.* (12). Moreover, the exceedingly lowered choline oxidase activity of liver homogenates from rats which had been on the carcinogenic diet for only a short time suggests that there may be interference in the transmethylation system in liver long before tumor development and that this interference may play a part in the onset of tumor growth.

SUMMARY

The rate of oxidation of choline at pH 7.8 in liver homogenates from rats on a carcinogenic diet containing 3′-Me-DAB has been found to be about 25 per cent of that in homogenates from normal or control rats. The retardation in activity was almost as great after 14 days of feeding 3′-Me-DAB as after more prolonged feeding. The activity in the liver tumor homogenates was less than 5 per cent of that in normal or control liver homogenates and less than 20 per cent of that in 3′-Me-DAB liver homogenates.

3′-Me-DAB liver or liver tumor homogenates, at pH 7.8 or 7.2, produced predominantly more betaine aldehyde than betaine as the end-products of the oxidation of choline, while normal or control liver homogenates produced only betaine. At pH 6.7, 3′-Me-DAB liver and liver tumor homogenates produced little or no oxidation of choline, even to the aldehyde, while normal and control liver homogenates produced both betaine aldehyde and betaine.

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Experimental Inhibition of Carcinoma by Lymphosarcoma*

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It has been our impression that patients with a lymphosarcoma rarely have an associated carcinoma. There are reports (8) which describe multiple primary malignant lesions, but the lymphomas, because of their "controversial nature," have been deliberately omitted from consideration. Of patients who die of malignant lesions, 2 to 4 per cent disclose multiple primary malignant lesions. From reports which do include the lymphomas, it is apparent that the combination of carcinoma with a lymphosarcoma is extremely rare.

Warren and Gates (9) studied 794 cases of multiple primary malignant lesions. Ten of these were of carcinoma associated with lymphosarcoma. Owen (6), in a study of 143 multiple malignant lesions, did not record a single association of the two types of neoplasm. Schreiner and Wehr (7) reported 307 cases of multiple primary malignant lesions, 4 of which showed a lymphosarcoma coupled with a carcinoma. Burke (1) analyzed data on 27 cases of primary multiple malignant lesions, none of which were associated with lymphosarcoma. The same was true for the 25 cases detailed by Kirshbaum and Shively (3).

Furthermore, the infrequency of metastatic involvement of the spleen by a carcinoma is a clinical observation which is pertinent to our inquiry. Willis (10) has stated that the incidence of splenic metastasis in malignant lesions which have come to necropsy has ranged from 1 to 4 per cent.

Experimentally, the controversial literature dealing with this presumed antagonism between the spleen and other lymphatic structures on the one hand, and the growth of a carcinoma on the other, has been reviewed by Woglom (11). In his study of 600 papers dealing with resistance to transplantable tumors, the role of the lymphocytes was extensively cited. It was shown by some that the development of immune reactions to transplanted tumors was always associated with the presence of lymphocytes. These cells were regarded, not as scavengers, but as an integral part in the development of this immune reaction. Some accessory factor was postulated, however, because satisfactory tumor growth was obtained in some rats with extraordinarily high lymphocyte counts; whereas others, with subnormal counts, were immune. Certain workers were quoted as having reduced the immunity of rats to tumors by lowering the lymphocyte counts with roentgen rays.

It would thus appear that there are conflicting opinions regarding the role of the lymphatic system in its association with carcinoma. One view is that the lymphatic system may be regarded as a sort of permanent "open house," facilitating the spread of the cancer cells. The contrary view holds that the lymphatic system is antagonistic to invading cancer cells and that it succumbs to them only after a struggle.

In view of the clinical observations recorded in the previous paragraphs and of the conflicting opinions which abound in the literature, we proposed an experiment whereby the influence, if any, exerted by a growing transplanted lymphosarcoma on the growth of a transplanted carcinoma, and vice versa, could be observed.

MATERIALS AND METHODS

In an initial study, 48 weanling male rats of the Sprague-Dawley strain were arranged into 4 groups of 12 rats each.

Group 1.—Each rat was given a subcutaneous injection, in the region of the thigh, of 0.5 cc. of a suspension of cells of Murphy-Sturm lymphosarcoma (5) in an isotonic saline solution.

Group 2.—Each rat was given a subcutaneous injection, along the back, of 0.2 cc. of a suspension of cells of the Walker rat carcinoma 256, in an isotonic saline solution.

Group 3.—Each rat was given injections of comparable amounts of the suspensions of both the lymphosarcoma and the carcinoma, subcutaneously, in regions identical to those selected for Groups 1 and 2.

Group 4.—Each rat was given a subcutaneous

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injection of 0.7 cc. of a mixed cellular suspension, composed of both the lymphosarcoma cells and the carcinoma cells, so prepared as to provide amounts of each tumor suspension comparable to those provided separately to animals comprising Group 3.

These tumors were measured in centimeters along their largest diameters on alternate days.

RESULTS

Group 1.—The Murphy-Sturm lymphosarcoma, after transplantation, exhibits a pattern of growth which is often followed by subsequent regression. One of us[1] has studied the growth patterns of this tumor and has observed this tendency toward regression. The percentage of tumors which regress, following a period of growth, is inversely proportional to the biologic age of the donor tumor. Whereas this tendency toward regression of transplanted tumors would be disappointing in certain types of experiments, it proved of interest to us in our present study. In our experience these lymphosarcoma transplants grew rapidly for 11 days after implantation and then regressed (Chart 1).

Group 2.—Transplants of the Walker carcinoma likewise grew rapidly and exhibited continuous growth until the death of the animal at about 23 days (Chart 1).

Group 3.—In this group of twelve rats, these tumors were implanted in different sites in the same animal. The growth patterns of the carcinoma and of the lymphosarcoma were essentially like those displayed by these tumors when they had been implanted into separate animals (Groups 1 and 2). The lymphosarcomas were not so large, however, and regression was obvious 2 days earlier than in those of Group 1 (Chart 1). In general it seemed obvious that each tumor had grown independently of the other and that the one had not exerted any significant influence on the growth of the other. These results, therefore, did not sustain the impression we had gained from reported clinical data that a natural antagonism between lymphosarcomas and carcinomas did exist.

Group 4.—The fourth group, in which the two tumor suspensions were thoroughly mixed and then implanted subcutaneously in amounts comparable to those which had been injected separately, presented some growth patterns which were of interest (Chart 1). Some of the tumors in this group grew rapidly following implantation and then regressed permanently. These tumors appeared to follow the growth pattern originally displayed by the lymphosarcoma implants. One group of five animals given the mixed suspension was of particular interest in that the implant continued to grow in a manner suggesting a pattern of growth characteristic of the lymphosarcoma. Following the growth period, there was a regression of each tumor, and in one case complete regression was observed (Chart 1). The regression of these five tumors was only temporary, however, and was followed in 4 days by a second period of uninterrupted growth.[2]

It seemed to us that the first two stages, those of growth and of subsequent regression, represented the period when the lymphosarcomatous constituents of the mixed suspension were dominant, since necropsy performed during this initial period of growth of some of the mixed implants showed that the tumors were almost exclusively composed of lymphosarcomatous cells. It was noted that sections of tumors obtained during the second phase of rapid growth—that which took place after the initial regression—were then very largely composed of carcinomatous cells. The cytologic structure was then predominantly typical of the Walker carcinoma. Since the initial growth was largely lymphosarcomatous and the second period of growth predominantly carcinomatous, an inhibitory effect of the former on the latter may be conjectured. It was not until regression of the rapidly growing lymphosarcoma

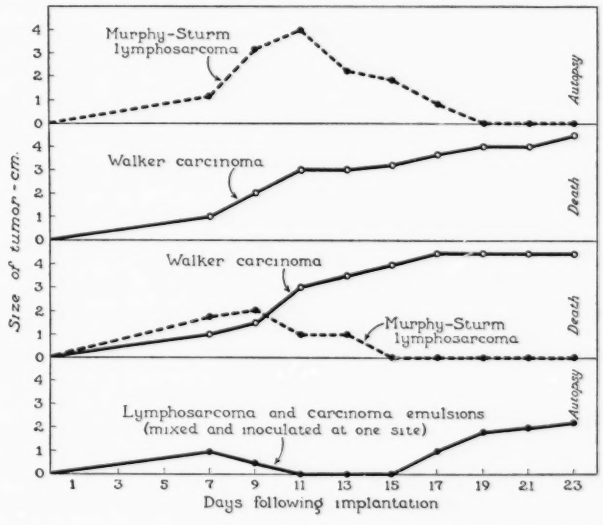

CHART 1.—Average sizes of tumors in centimeters, in four groups of animals: (*a*) lymphosarcoma alone; (*b*) Walker carcinoma alone; (*c*) carcinoma and lymphosarcoma in separate sites in the same animal; (*d*) carcinoma and lymphosarcoma implanted together into the same site of each animal.

[1] T. C. Donald, Jr., unpublished data.

[2] Note that the first three curves in Chart 1 represent averages. The fourth curve represents the most striking case in this group. In the other 4 cases mentioned in Group 4, the temporary regression was not complete.

had occurred that the extensive growth of the carcinoma took place.

Since the initial experiment showed that, when mixed suspensions were implanted the growth of the carcinoma was invariably delayed, we repeated our study. Forty young rats were selected and arranged into four groups of ten animals each, and implantations of cellular suspensions were made as before. Results of the second experiment were essentially the same as those obtained in the first. The combined survival data in the two experiments 23 days after implantation have been condensed into Table 1. Of the 22 animals given a

TABLE 1

RESULTS AT THE END OF 23 DAYS, WITH 22 ANIMALS IN EACH GROUP

	Living		Dead	
	No.	Condition	Examined at necropsy	Of tumor
Lymphosarcoma	11	1 no take 10 regressed	5	6
Carcinoma	4	1 regressed 3 growing	3	15
Both, different sites	2	1 regressed 1 (one tumor growing)	3	17
Both, same site	12	4 regressed 8 growing	5	5

lymphosarcoma implant, 11 were living. Of these, the implant in 1 had failed to grow and in 10 all tumors had regressed. Of the 11 dead, 6 had died from the effects of the tumor and 5 had been killed to obtain viable tissue for study. Of the 22 animals inoculated with cellular suspensions of the carcinoma, 4 were living. One tumor had regressed, but 3 were growing; 15 animals had died, and 3 had been killed to obtain tissue. In Group 3, only 2 animals had survived, 17 had died, and 3 had been killed. In Group 4, animals which had received mixed suspensions of both tumors, 12 were still living and only 5 had died from the effects of the tumor. This group also had a greater number of growing tumors than any other group at the end of 23 days.

It appears from these data that the lymphosarcoma may have moderated the degree of malignancy of the carcinoma in those animals into which the mixed suspensions of these two types of neoplastic tissue had been injected. The separate implantation of cellular suspensions of these two tumors into different sites in the same animal had failed to reveal any such attenuated pattern of response of the carcinoma. Furthermore, we did not observe any significant difference between the growth patterns of these two tumors when they were implanted into the same animals (Group 3) and when they were implanted separately, as in Groups 1 and 2.

By the 23d day following implantation, 15 (68 per cent) of the 22 animals that were treated with carcinoma alone (Group 2) had died of the lethal effects of the tumor, and 17 (77 per cent) of the 22 animals inoculated with both carcinoma and lymphosarcoma in separate sites (Group 3) had died of the effects of the tumor. But 6 (27 per cent) of the 22 animals bearing the implanted lymphosarcoma had died, and only 5 (23 per cent) of those bearing the mixed tumor cell growths had died. When these mortality data are contrasted with those set forth previously—68 per cent in Group 2 and 77 per cent in Group 3—it is obvious that some inhibitory influence, presumably exerted by the lymphosarcoma on the carcinoma, extended survival times of animals of Group 4.

COMMENT

One may postulate from the data assembled on the growth of implants within Group 4 that, when suspensions were mixed and implanted into the same site, the organism permitted only one of the two neoplasms to demonstrate detectable growth at any one time. It may be that cellular constituents of the one neoplasm were destroyed or kept in abeyance; or that growth factors essential for its nutrition were withheld. It may well be that certain enzyme systems either in the host or in the tumor tissue play a part in the growth of both the lymphosarcoma and the carcinoma cells, favoring at times lymphosarcoma cells when both lymphosarcoma cells and carcinoma cells are present in suitable numbers for individual growth. An analogous situation is thought to occur in diabetes. The oxidation of fats is accomplished by the same enzyme system, which, in the normal nondiabetic animal, would favor the oxidation of carbohydrates (4). And, again, when para-aminobenzoic acid is present, a micro-organism such as Staphylococcus will take it up and thrive. But, when sufficient sulfonamide is available, it will be favored by the same enzyme system hitherto allied to the micro-organism, and thus to the latter's detriment (2). Bacteriologists call this "competitive inhibition." Perhaps there is a substance in the lymphosarcoma cell which competes successfully for the enzyme system of the host essential for the growth of carcinoma when both are trying to grow at the same site. When the lymphosarcoma regresses, the enzyme system could then become available for the growth of carcinoma.

SUMMARY AND CONCLUSIONS

A study has been made of the effects obtained in experimental animals when suspensions of lym-

phosarcomatous cells and carcinomatous cells are implanted, separately and in mixtures.

The results showed that, of 22 rats implanted with lymphosarcoma, tumors grew rapidly in 21 animals for a certain period of time and then regressed. All tumors grew rapidly in the 22 animals which received implants of the carcinoma. When cell suspensions were implanted in separate sites on the same animal, the resulting tumors followed growth patterns which were more or less like those obtained when implantations were made into separate animals. When mixed suspensions of the two tumors were implanted, however, tumor growth patterns suggesting certain inhibitory relationships were seen. A period of rapid growth, a period of regression, and a subsequent period of growth were observed. Sections of the tumor obtained during the initial growth showed that the tissue was predominantly lymphosarcomatous, whereas sections obtained during the second period of rapid growth showed that the tissue was then predominantly carcinomatous.

Our study would seem to support the opinion that lymphosarcoma and carcinoma, when growing independently in separate locations in the same animal, display no antagonistic growth patterns. However, when lymphosarcoma cells and carcinoma cells are implanted together and subjected to identical environmental factors provided by the host, there appears to be a preferential development of the lymphosarcoma. Significant growth of the carcinoma cells did not take place until growth and regression of the lymphosarcoma had occurred. Possible explanations for this sequential development are offered.

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Catalase Studies on Protein-depleted Rats Bearing the Jensen Sarcoma*

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The effect of tumor growth on the liver catalase activity of rats on normal diets has been well established (1, 4, 6), and some work has been performed at low and high dietary protein levels (6). The investigations have not indicated that increased dietary protein will alleviate the condition of decreased liver catalase activity induced by tumor growth. This would suggest that nutritional factors were either nonoperative or that the effects of the tumor on liver catalase activity were so large in proportion to the nutritional effects that the latter would be difficult to detect. Increased levels of dietary protein would also be more favorable to tumor growth and thus increase its effect on liver catalase. The present experiments were undertaken in an attempt to minimize the variability of dietary protein intake by the simple expedient of a protein-free diet and to determine the effects of tumor growth on liver catalase activity under these conditions. The combined effects of protein depletion and tumor growth should lead to a maximum of interference with the mechanism for synthesis of catalase and produce lower levels of liver catalase activity than under normal dietary conditions. The maintenance of normal protein-depleted levels of liver catalase activity in the presence of tumor growth would be an indication of either the ability of the animal to synthesize catalase despite protein depletion or the relatively minor effect of the tumor on liver catalase activity under these conditions.

MATERIALS AND METHODS

Adult rats of both sexes of a Long-Evans black-hooded strain were used for all the experimental work. Individual groups were usually selected so that all animals were the same age, within close limits. Before the tumor implantations the animals were normally maintained either on a routine diet of Rockland pellets with supplements of lettuce twice-weekly or entirely on the protein-free diet described in a previous publication (1). The methods of tumor transplantation were similar to those reported by Dunn *et al.* (2).

The animals were subjected to the three different types of treatments described below:

1. Active sarcoma transplants were implanted subcutaneously into rats maintained on a normal diet until definite small amounts of tumor growth (4–8 gm.) were evident. This occurred approximately 12 days after implantation, and at this time the animals were placed upon the protein-free diet.

2. The animals were maintained on the protein-free diet continuously. Implantation of the tumor occurred after 21 days of protein depletion.

3. The tumor was implanted after 10 days on the protein-free diet. The same diet was maintained for 8 additional days until evidence of tumor growth (2–4 gm.) was obtained. At this time the animals were given a diet of pure casein with twice the vitamin supplement used in the protein-free diet.

The preparation of the tissues for analysis, the determination of blood and erythrocyte volumes, and the methods of catalase and nitrogen determinations employed for the liver and kidneys are described in detail in a previous publication (1). Catalase activity is expressed in terms of an arbitrary unit—a unit consisting of that amount of catalase which will liberate 1 ml. of oxygen per second from a 1.0 N H_2O_2 solution at 0° C. and a pH of 7.0.

RESULTS

The pertinent data on the three experimental groups of animals are presented in Tables 1–4. Comparative data are also presented showing the effect on levels of enzyme activity of dietary factors independent of tumor growth. Enzyme activities are calculated on a unit nitrogen basis and per unit total weight of the animal.

Protein-depleted animals.—The growth of tumor in protein-depleted rats produced a slight decrease

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TABLE 1

THE EFFECTS OF THE JENSEN SARCOMA ON PROTEIN-DEPLETED RATS*

Days since implant†	2	5	7	13	17	19	20	20	21	21	21	25	29	Control 40 days protein-free diet	Av. value protein-free diet
Total wt., gm.	169	200	170	150	160	151	150	185	134	150	155	147	147	153	
Tumor, wt., per cent	(trace)		1.0	3.9	7.1	17	1.5	0.4		(trace)		12	10	0	
Per cent wt. loss since implant	0	1.5	−5	9.1	11	15	14	15	22	14	17	15	16	14	
Blood volume ml/100 gm total wt	5.9	5.5	5.4	6.7	5.9	5.8	5.4	5.7	6.1	5.3	5.9	6.7		5.6	5.7
RBC volume ml/100 gm total wt	2.3	2.3	2.3	3.2	2.2	1.8	2.1	2.4	2.1	1.9	2.3	1.7		2.2	2.2
Liver N mg/100 gm total wt	119	89	120	85	103	119	87	84	98	83	99	105	88	82	87
Liver catalase activity units/100 gm total wt‡	282	290	326	238	220	194	190	256	318	312	326	209	96	248	265
Liver catalase activity units/mg liver N	2.36	3.24	2.73	2.82	2.17	1.63	2.18	3.04	3.23	3.78	3.29	2.01	1.10	3.04	3.02
Kidney catalase activity units/100 gm total wt	24	28	27	31	47	43	44	63	33	46	41	51	41	33	39

* Data represent individual animals.

† All female animals (10 months old), tumor implanted after 21 days of protein-free diet.

‡ Catalase activity unit equals 1 ml. oxygen per second from a 1.0 N H_2O_2 solution.

TABLE 2

THE EFFECTS OF A PROTEIN-FREE DIET ON RATS WITH THE JENSEN SARCOMA*

Sex (10 months old)	F	F	F	M	M	F	F	F	F	Av. value protein-free diet
Days since implant	14	14	16	14	14	19	20	20	24	
Days protein-free diet and tumor growth	2	2	4	4	4	7	10	10	12	
Total wt., gm.	203	204	186	338	342	204	198	243	178	
Tumor, wt., per cent	8.9	4.4	7.0	0.5	6.3	7.3	1.5	5.8	20	
Per cent wt. loss on protein-free diet	4	7	10	10	6	9	17	16	18	
Blood volume ml/100 gm total wt	6.3	5.0	6.8	5.6	5.8	5.9	6.2	7.2	8.2	5.7
RBC volume ml/100 gm total wt	2.1	2.0	3.1	2.7	1.9	2.3	2.8	2.3	1.7	2.2
Liver N mg/100 gm total wt	107	118	109	97	108	101	102	132	143	87
Liver catalase activity units/100 gm total wt	222	300	253	213	230	216	195	204	374	265
Liver catalase activity units/mg liver N	2.17	2.54	2.34	2.20	2.12	2.14	1.91	1.55	2.61	3.02
Kidney catalase activity units/100 gm total wt	36	31	26	43	32	28	27	19	14	39

* Data represent individual animals.

TABLE 3

THE EFFECTS OF 100 PER CENT CASEIN ON PROTEIN-DEPLETED RATS WITH THE JENSEN SARCOMA*

Sex (12 months old)	F	F	F	F	M	F	F	F
Days since implant	12	15	15	30	26	18	18	18
Days protein-free diet	18	18	18	42	29	18	18	18
Days 100 per cent casein	4	7	7	7	7	10	10	10
Total wt., gm	220	172	186	165	176	176	167	169
Tumor, wt., per cent	3.7	4.4	9.7	7.6	5.7	18	16	12
Per cent wt. gain or loss on casein	1	−6	−8	1	−16	−9	−16	−13
Blood volume ml/100 gm total wt	4.9	6.0	5.8	5.9	5.4	8.5	6.5	7.5
RBC volume ml/100 gm total wt	2.0	2.6	1.7	2.4	1.1	1.9	2.7	2.7
Liver N mg/100 gm total wt	117	154	128	153	181	158	91	154
Liver catalase activity units/mg liver N	3.18	3.54	2.40	3.72	3.61	0.93	1.54	1.70
Liver catalase activity units/100 gm total wt	370	546	307	566	653	147	139	262
Kidney catalase activity units/100 gm total wt	60	63	64	69	89	27	27	43

* Data represent individual animals.

in liver catalase activity which seemed to be related to the approximate size of the tumor, i.e., a drastic decrease was only observed if at least 10 per cent tumor growth occurred. In most cases animals bearing tumor implants for a maximum period of 25 days showed no greater than a 25 per cent decrease in liver catalase activity below the level in protein-depleted animals. In many cases the kidney catalase activity was above the normal value in the tumor-bearing animals. There is some indication that the presence of tumor increases the liver nitrogen above the level which occurs in the protein-depleted rat.

Protein depletion after tumor establishment.—The lowering of liver catalase activity by tumor establishment followed by a protein-free diet leads to approximately the same level of liver catalase activity as the growth of tumor in previously protein-depleted animals, i.e., a 25 per cent decrease below the level in protein-free rats. Relationships between the amount of tumor growth or its rate of growth seem to have little difference in ability to lower the liver catalase activity below a value approximately 40 per cent of the activity of the normal animal. In fact, the animal with the largest amount of tumor has the highest liver catalase activity and the highest value for liver nitrogen. In general, these animals have a higher level of liver nitrogen than is found in the protein-depleted rat. The effect of these conditions seems to be more drastic on kidney catalase activity than on liver catalase.

TABLE 4

THE EFFECT OF VARIOUS PROTEINS ON RECOVERY OF CATALASE ACTIVITY IN PROTEIN DEPLETED RATS

Protein source	Weight per cent in diet	Days on diet	Catalase per cent of normal	
			Liver	Kidney
Casein	15	2	75	90
Casein	15	5	100	100
Gluten	15	13	75	100
Gluten	30	17	80	160
Gelatin	60	9	50	110
Gelatin	90	11	90	230

Tumor establishment followed by protein repletion.—The establishment of tumor in protein-depleted rats which were then fed a diet of 100 per cent casein demonstrates that catalase may be increased above the protein-depleted level in the presence of tumor growth. The effect of the tumor is evidently quantitative, i.e., large amounts of tumor or rapid neoplastic growth eventually lead to lowering of liver catalase, but in the early stages of protein repletion catalase increases. This increase is even more striking in the case of the kidney, in which was found an initial elevation of the kidney catalase activity above the value found in normal rats without tumor growth. Liver nitrogen in many instances is elevated above the value found in normal animals and in animals maintained on 100 per cent casein without tumors. Blood volume relationships indicated increased plasma volumes concomitant with increased amounts of tumor growth under all the experimental conditions. These data are similar to the results obtained for blood volumes of tumor-bearing rats on a normal diet (1, 3).

DISCUSSION

It is believed that the data demonstrate that, although the tumor has the ability to lower liver catalase activity, it does not interfere with the synthesis of the enzyme. This is indicated by the initial ability of the protein-depleted, tumor-bearing rat to exceed the protein-free level and approach the normal liver catalase level when supplied with a 100 per cent casein diet. Thus, the possibility of competition between the tumor and the host for a component essential to catalase synthesis may exist. This, in turn, is related to the quantitative demands of the tumor. The ability of the tumor to interfere with kidney catalase activity is also demonstrated on a 100 per cent casein diet, but the effect of the casein diet has been to keep the kidney catalase activity at a much higher level than in rats on a normal diet. When drastic decreases in liver catalase activity have occurred, values below the kidney catalase activity in normal rats would be expected, but the casein diet seemed to prevent such a lowering of activity. However, the tumor did lower kidney catalase activity below that of control animals on a 100 per cent casein diet when tumor growth was sufficient to lower liver catalase activity.

The suggested elevation of kidney catalase activity in protein-depleted tumor-bearing rats is not readily explained. This may be caused not by the tumor but by placing the animal on a relatively high protein diet after a prolonged period on a protein-free diet. It is also possible that catalase synthesis in the kidney is accomplished by a different mechanism than in the liver. Some support for these suggestions is found in some additional data (Table 4) from this laboratory. These observations are the results of a preliminary study on the rate and extent of recovery of liver and kidney catalase of protein-depleted rats which have been fed diets containing proteins from three different sources. It is evident that, while 15 per cent casein restores liver and kidney catalase in a few days, gelatin in very high concentrations does not restore liver catalase but causes an increase to more than twice

the normal value in kidney catalase. Gluten seems to have an intermediate effect. It may be profitable to explore this subject further.

The slightly higher liver nitrogen level in the protein-depleted rats with tumor, as compared to that in protein-depleted animals, suggests that the tumor may stimulate incorporation of nitrogen into the liver even in the absence of a dietary source of nitrogen. Higher levels of liver nitrogen are established under normal nutritional conditions in chick embryos (5) and in rats bearing tumors (1).

SUMMARY

Liver catalase activity is decreased by the growth of the Jensen sarcoma in protein-depleted rats, but the decrease is not so severe as that achieved in tumor-bearing rats on a normal diet. This may be related to the smaller amount of tumor growth in the former. Protein-depleted rats with tumor show evidence of liver and kidney catalase synthesis in the presence of tumor when supplied with a diet of 100 per cent casein. Normal levels of liver catalase activity with appreciable tumor growth are attained initially but are eventually lowered to levels characteristic of animals bearing rapidly growing Jensen sarcoma. Kidney catalase activity of normal or tumor-bearing rats is elevated by a 100 per cent casein diet above that of rats on a normal protein diet.

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The Nature of the Antigen in Induced Resistance to Tumors

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The mutual interplay of biological forces between a tumor and its host, which may, according to conditions, promote, restrict, or prevent the growth of a tumor, constitutes what is usually meant by the term "tumor immunity." The broad field covered by that term can be divided into several special subdivisions, of which "induced resistance" is one. The latter term was used by the early workers in this field when referring to the induction of resistance to the implantation of tumors by the prior inoculation of normal homologous cells. It is with that phenomenon that the present communication is primarily concerned.

The vast background of literature related to tumor immunity has been thoroughly reviewed by Woglom (13), Spencer (11), and Snell (10) and need not be gone into here, except for selected relevant papers. Little will be said here outside of the field of nonspecific induced resistance to transplantable tumors, i.e., work in which normal tissues were used as the antigen.[1]

The earliest observations directly related to the work reported here were made by Bashford, Murray, and Cramer (2) who demonstrated that resistance to the subsequent implantation of tumors could be induced by the prior inoculation of normal tissues, including defibrinated blood. They also said that in this reaction the cells were active and the serum was not. Subsequently, Haaland (3) adduced evidence that "devitalization" of normal (or neoplastic) cells ablated their immunizing power. These early results suffered from the fact that they were obtained in "stock" animals, a defect which at that time was universal. Most of the papers on the general subject of tumor immunity were written prior to the time when genetically controlled tumors and animals came into general use, and it is now known that many of the older observations could be produced at will by a judicious selection of the materials according to their genetic make-up.

In more recent years it was reported that induced resistance to transplantable leukemia could be elicited by normal spleen or lymph node (9) and by embryonic tissues (7). In the latter case genetic constitution exerted an important influence upon the results. Several years ago the senior author began to reinvestigate this problem, using defibrinated blood as the antigen and carcinomas as test tumors (1). It was established that the primary factor, but not necessarily the only factor, in induced resistance was the genetic relationships between the three biologic components of the system (i.e., the host, the tumor, and the blood), at least within the limits of the materials used. A spectrum of results could be obtained, ranging from complete susceptibility to nearly complete resistance, depending upon how the materials were selected with regard to genetic origin. About 3 years ago a new tumor was obtained from Dr. H. B. Andervont. This unusual tumor is a sarcoma which originated in strain DBA but grew progressively in a high percentage of strain C mice in the first transplant generation. An opportunity was thus provided to study the influence of experimental modifications of the defibrinated blood while maintaining close genetic control of the tumor, the host, and the source of the blood. The results obtained form the basis for this report.

MATERIALS AND METHODS

General Experimental Conditions and Materials

The basic features common to all experiments will be described first; the special technical variations, together with their results, will follow.

The hosts in which resistance was tested were of two inbred strains, DBA and C (B alb C), both of the Heston sublines and both described by Law (6). The hosts varied in age from 7 to 16 weeks,

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[1] To avoid more cumbersome terms, the term "antigen" is used hereafter to indicate a substance capable of inducing a state of immunity without reference to the presence or absence of demonstrable antibodies.

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and both sexes were used. Distribution with regard to sex and age was approximately equal throughout the experiments. Groups of ten animals were housed on wood shavings in plastic cages in an air-conditioned room. Purina Laboratory Chow and tap water were supplied ad libitum.

The tumor, which is referred to as sarcoma DBA 49, arose spontaneously in the region of the right anterior mammary gland in a strain DBA female in the colony of Dr. H. B. Andervont, who found that, as expected, it grew in all strain DBA mice, but, unexpectedly, it also grew in a high percentage of strain C mice and in a few mice of other strains. Through the generosity of Dr. Andervont we obtained the tumor, and our experience paralleled that of Andervont in detail. It was determined that the tumor grew in approximately 90 per cent of strain C mice with only an occasional regression and attained a size of approximately 1.5×1.0 cm. in 3 weeks. Metastases have not been observed by gross inspection during the course of this work. The stock tumor, from which material for transplantation was taken at irregular intervals between the 8th and 34th generations, has been grown in strain C mice continuously since the first transplant generation. Dr. Thelma B. Dunn, to whom the authors are grateful, has examined sections of this tumor and described it as a fibrosarcoma of the ordinary type, composed of interlacing bundles of spindle cells showing moderate anaplasia. In a strain DBA mouse bearing a transplant of the original tumor, Dr. Dunn has seen a metastasis in the lung.

Two methods for transplanting the tumor were used in some of the early experiments: one, the common trocar method, has been described in a previous publication (1). The second, employing a brei, has been found to be less time-consuming and rather more accurate as to dosage. In this method clean, viable tumor tissue was dissected free from any adhering normal or necrotic tissue and passed through a simple tissue press. The resulting tumor pulp was thoroughly mixed with an equal quantity of normal saline and loaded into a tuberculin syringe with a 22-gauge needle. A dose of 0.025 cc. of the 50 per cent brei was deposited beneath the skin in the region of the right flank of the host. Aseptic precautions were employed throughout the procedure. No observable change in the data resulted from the change in method, and, at the time when the last set of experiments was done, the trocar method was abandoned.

In all cases the starting material for the antigen was blood taken from the surgically exposed hearts of adult mice under light ether anesthesia. The blood was withdrawn aseptically by means of a syringe moistened with saline and transferred to a flask where it was immediately defibrinated by gentle agitation with glass beads. The defibrinated blood was then subjected to special conditions or procedures to be described later. Whatever the state or dilution of the blood, a final volume of 0.2 cc. (in a few cases to be noted later the conditions were such as to make 0.1 cc. more suitable) was deposited beneath the shaved dorsal skin of the test animals by means of a syringe and a fine needle. After the injection had been made, the needle tract was pinched with fine forceps as the needle was withdrawn and was held while the point of insertion was touched with flexible collodion. If this procedure is followed, minimal backbleeding occurs.

At the end of an incubation period of 21 days the tumor was implanted, and the animals were observed for the presence of an established graft at weekly intervals for 4 weeks. The tumors, when present, were measured by means of calipers and the measurements recorded. The data are based upon the number of positive cases at 3 weeks.

Special Experimental Conditions and Results

The first series of experiments was designed to test the susceptibility of this sarcoma to resistance induced by homologous normal tissues and to extend the previous observations with regard to strain specificity of the reaction. Four independent experiments were done on mice of each of the two strains, divided into control and test groups. The controls were given an injection of 0.2 cc. of normal saline. The test animals of each strain were given 0.2 cc. of defibrinated blood of the alternate strain. After the incubation period all the animals were given inoculations of the tumor and were observed for 4 weeks. As expected, C blood did not induce resistance in DBA mice against the DBA tumor; the tumor grew in all of 76 control mice and 51 test mice of strain DBA. However, in C mice receiving DBA blood a high degree of resistance was induced, there being only 6 per cent of test animals with an established graft, compared to 88 per cent in the control animals (Table 1). Statistical analysis indicates that the difference between the control and test groups of strain C is highly significant ($P < 0.001$).

Next, a comparison of the effectiveness of serum and cells was undertaken. In this and the succeeding experiments only strain C mice were used as hosts. Defibrinated blood from strain DBA mice was prepared as before and centrifuged at 2,000 r.p.m. for 10 minutes at 0° C. in a refrigerated centrifuge with a horizontal head. The supernatant serum was removed and set aside in the refrigera-

tor. The cells were washed by twice resuspending in saline and recentrifuging. Finally both the serum and the cells were diluted with saline to the original volume of the defibrinated blood. Each of the antigens was injected in 0.2-cc. doses into a group of strain C mice and the mice tested for susceptibility or resistance to the tumor in the usual way. Three such experiments were done independently. The combined results of these three experiments are shown in Table 1. The probability that this difference could have been obtained by chance is much less than 0.001. It seems quite clear that the active fraction is in the cellular elements (or in an easily sedimentable element) and not in the serum.

TABLE 1

RESISTANCE TO THE DBA TUMOR DEVELOPED IN STRAIN C MICE IMMUNIZED WITH STRAIN DBA BLOOD, SERUM, OR CELLS

Antigen*	No. hosts	No. with tumor	Per cent positive
Saline	50	44	88
Blood	50	3	6
Serum	57	46	81
Cells	54	3	6

* Dose equivalent to 0.2 cc. defibrinated blood.

Although it had already begun to appear that the red cell was an effective antigen in the phenomena under study, this point has been disputed (4), and it appeared desirable to perform a more complicated separation of the constituents of the blood than simple centrifugation. The method of Vallee *et al.* (12) was adopted. Bovine albumin (30 per cent, Armour & Co.) was carefully diluted with saline to obtain a solution having a specific gravity of 1.080, determined gravimetrically by pycnometer. Defibrinated blood from strain DBA mice was layered on top of the albumin in a centrifuge tube, and the mixture was spun at 800 r.p.m. for 10 minutes and then at 3,000 r.p.m. for 30 minutes in a refrigerated centrifuge at 5° C. After removal from the centrifuge four layers were carefully removed, namely, serum, white blood cells, albumin, and red blood cells, in that order. None of these layers was entirely pure. A drop of each was inspected under the phase microscope and in stained smears. Each cellular layer contained a few cells of the alternate type, and each clear layer contained a few cells of both types but never a sufficient number to affect the outcome when considered from the standpoint of dosage as determined in another series of experiments. No platelets were identified, but it can be safely assumed that the few platelets that might have escaped the defibrination process would, because of their specific gravity, be found either in the leukocyte layer or above it, but not in the red cell layer. The three blood-derived fractions were diluted with saline to the original volume of the blood, and the albumin fraction was diluted to produce a total protein content similar to that of serum. The power of each to induce resistance at a dosage of 0.2 cc. was compared in the usual way with the power of an aliquot of untreated blood that had been set aside during the procedure. The experiment was done 3 times with the same outcome, and the results (Table 2) show that a suspension of red cells induces a degree of resistance which is comparable to that produced by defibrinated blood.

These results suggested an investigation of the influence of dosage. Accordingly, a set of experiments was done in which graduated doses of defibrinated blood and equivalent doses of washed cells were compared. (All doses mentioned hereafter will be equivalent doses, i.e., the amount of defibrinated blood from which the antigen was derived rather than the actual volume given.) Blood from strain DBA mice was prepared as before and separated into two equal portions. Washed cells were prepared from one portion and resuspended in the original volume of saline. Serial twofold dilutions of both aliquots were then made, and each dilution was administered to a group of strain C mice that was later inoculated with the tumor. Two such experiments were done with results which show that (*a*) the degree of resistance induced varies with the dose of either blood or cells, (*b*) the dose which will reduce the percentage of successful grafts by more than half (from 88 to 30 per cent) lies somewhere in the neighborhood of 0.0125 cc., and (*c*) a significant degree of resistance (52 per cent successful grafts) can be induced by as little as 0.0031 cc. of blood or cells.

TABLE 2

DEGREES OF RESISTANCE INDUCED IN STRAIN C MICE BY FRACTIONS OF STRAIN DBA BLOOD

Doses are equivalent to 0.2 cc. of defibrinated blood. Number of successful grafts is shown by the numerator; number of mice inoculated is shown by the denominator.

Exp. no.	Successful grafts after immunizing with: Blood	RBC	WBC	Serum	Albumin
1	5/19	6/17	13/18	15/19	11/15
2	1/19	1/17	6/19	18/18	12/15
3	3/19	4/16	6/15	12/16	18/20
Total	9/57 (16 per cent)	11/50 (22 per cent)	25/52 (48 per cent)	45/53 (85 per cent)	41/50 (82 per cent)

Therefore, a dose of 0.02 cc. was adopted for further work.

Thus far the experiments serve primarily to establish a background for the following experiments with laked cells. Various methods were explored until, by trial and error, three suitable technics for laking the cells were found. The first was slow freezing. A cell suspension was placed in the ice compartment of the refrigerator at −15° C. for 1 hour and immediately spun for 10 minutes in a centrifuge at room temperature, whereupon most of the cells were laked. When the partially laked suspension was refrozen and again centrifuged only a few cells escaped destruction.[2] Our experience suggests that the efficiency of this procedure depends, in part at least, upon centrifuging while thawing takes place. With the use of this freezing method, two experiments were run in which a frozen cell suspension was compared with a control suspension kept unfrozen in the food compartment of the refrigerator at 3° C. The equivalent dose was 0.02 cc., and the animals were tested as usual. The results (Table 3) show that freezing abolished the power of the cells to produce resistance. The unfrozen cell suspension produced a degree of resistance in keeping with the dose, but the animals treated with frozen cells did not differ from nonresistant strain C mice. The difference between the two groups is highly significant statistically ($P < 0.001$).

A second relatively simple method of breaking up the red cells—laking with water—was next employed. A suspension of washed strain DBA red cells was prepared as before and equally divided into two centrifuge tubes. Both were centrifuged in the cold and the supernatants removed. The control aliquot was resuspended in 10 volumes (referred to the original volume of the blood) of saline, and the other was resuspended in 10 volumes of cold (ca. 5° C.) distilled water. Laking occurred in the latter in a few seconds, and dry salt was immediately added to restore normal tonicity. Examination of a drop of the laked suspension under the phase microscope revealed very few cells of any kind; there was, however, a small number of red cells, "ghosts," and white cells. Both suspensions were then used in the usual way to produce resistance in strain C mice. The equivalent dose was 0.02 cc. Three such experiments were done, and the combined data are shown in Table 3, in which it can be seen that the laked cells produced no resistance, but the intact cells produced a degree of resistance proportionate to the dosage and handling of the cells. The difference observed is highly significant statistically ($P < 0.001$).

A third physical principle was used to break up the cells, namely, exposure to high-pitched sound waves—high-pitched but not supersonic. It was found that nearly complete laking could be accomplished by exposing a suspension of red cells in 5 volumes of saline to sonic vibrations at a frequency of 9 kilocycles with 110 volts input for 90–120 seconds.[3] The cell suspension was contained in a thoroughly washed Lusteroid tube which, in turn, was immersed in the distilled water filling the cup of the instrument. Heating was prevented by circulating water at 2° C. A suspension of strain DBA red cells was prepared as before and divided into two parts. The test aliquot was subjected to the conditions just described. The control aliquot was subjected to the same conditions in every detail, except that the instrument was not turned on. Both portions were then tested (equivalent dose, 0.02 cc.) for their power to induce resistance in strain C mice by the usual method. Three such experiments were done, and the combined results (Table 3) indicate that the mice given sonically laked cells were nonresistant, whereas the mice given the control suspension were resistant. The difference observed is highly significant statistically ($P < 0.001$).

TABLE 3

EFFECT OF LAKING ON THE ABILITY OF RED CELLS TO INDUCE RESISTANCE TO STRAIN DBA TUMOR IN STRAIN C MICE

Immunizing injections were saline suspensions of washed cells from 0.02 cc. of strain DBA blood

Treatment of cells before injection	No. hosts	No. with tumor	Per cent positive
None (unlaked controls)	204	66	32
Laked by freezing	58	53	91
Laked by water	59	53	90
Laked sonically	55	50	91

Because of the possibility that when the cells were broken up by one of these methods some inhibitory substance or autolytic enzyme was released and interfered with the development of resistance, the experiment tabulated in Table 4 was done. The technical details are exactly like those of the immediately preceding experiments, except that in this case one of the antigens contained only sonically laked cells, whereas the other contained equal parts of laked and unlaked cells. Both anti-

[2] In these experiments, and in other similar situations, the residual cells and cell debris were not removed but were resuspended. A drop of the suspension was removed for examination under the microscope, but except for that the injected antigen always represented the entire contents of the aliquot and not just the clear supernatant fluid.

[3] The instrument used was a Raytheon Type R-223, and the input voltage was read on the input variac.

gens were given in 0.2-cc. doses and tested as usual. The results show that the intact cells induced resistance in proportion to the dose, and there is no evidence that the laked material inhibited the reaction.

DISCUSSION AND CONCLUSIONS

The results of the first series of experiments confirm some of the principles set out in the earlier publications of Bashford *et al.* and of the senior author. That is, a high degree of resistance against the implantation of a tumor can be elicited by the prior injection of normal, homologous, defibrinated blood. However, when methods available at the present are used, resistance of this type cannot be induced in an inbred mouse against a tumor that originated in the same strain.

TABLE 4

EXPERIMENT TO TEST FOR PRESENCE OF INHIBITORS IN LAKED CELLS. NUMERATOR EQUALS THE NUMBER OF SUCCESSFUL GRAFTS; DENOMINATOR EQUALS THE NUMBER OF MICE INOCULATED

Products of laking did not inhibit the power of the intact cell to induce resistance. The difference is highly significant ($P < 0.001$).

SUCCESSFUL GRAFTS AFTER IMMUNIZING WITH	
0.2 cc. laked cells	0.1 cc. laked cells plus 0.1 cc. intact cells
20/20	3/20
100 per cent	15 per cent

There is one point regarding the tumor that is worthy of note. Previously, there had been some question as to whether sarcomas were susceptible to this type of resistance (13); there can be no question that this tumor is a sarcoma and is susceptible.

The results of the second set of experiments show that the immunizing activity of defibrinated blood resides in the cells, while serum is essentially inert. The percentage of successful grafts following immunization with serum and cells approximate those in nonimmune and immune mice, respectively. These results with inbred animals confirm the conclusion reached by Bashford *et al.* with non-inbred animals. The principal importance of this is that it is the first step in simplifying the immunizing agent. It may be well to remark that there have been several indications that the presence of serum has a protective effect on the cells and that when this effect is present the antigenicity of the cells may not be entirely abolished by relatively gentle procedures, particularly in the higher dosages. Furthermore, as the cells are separated from the serum and subjected to much handling, they may undergo some slight injury so that their full power to induce resistance is reduced, particularly in the lower dosage range.

The experiments on dosage were not intended to be a complete and accurate investigation of this phase of the problem. Accurate determination of dosages would involve an amount of work that is not justified by present needs. Even with rough outlining of the influence of dosage, the data indicate that the degree of resistance produced is related to the dosage, whether defibrinated blood or washed cells is injected. Furthermore, a statistically significant degree ($P < 0.01$) of resistance was observed in animals that had received as little as 0.0031 cc. (equivalent dose) of defibrinated blood or cells. It is noteworthy that this dose is 100 times smaller than the dose thought necessary by the early workers in this field (2). From the approximate data and from our experience throughout the work it also appears that, in general, the degree of resistance produced by a given dose of washed cells is somewhat less than that produced by the equivalent dose of defibrinated blood, probably because minor injury is done to the cells by handling.

In evaluating and interpreting the data in Table 2 it should be remembered that it was not desired to show that the white cells would not produce resistance but rather to show that the red cells would do so. It is in keeping with the working hypotheses employed in designing these experiments to assume that almost any intact homologous cell might pro luce resistance and that this property would be shared by the white cells in some degree. In fact, Itami (4) has stated that white cells would induce resistance but red cells would not. Our data indicate that the white cell did, indeed, have power to produce resistance, and the degree of resistance produced was in keeping with the number of cells administered. Attention should be called to the fact that, when white cells were administered in a dosage equivalent to 0.2 cc. of undiluted blood, approximately half of the animals were resistant (this would be about the same as the resistance seen after approximately 0.005 cc. of red cells), and the small number of white cells present in the serum and albumin gave rise to no significant resistance. The equivalent dose used in most of these experiments was only one-tenth of the 0.2-cc. dose, and that is the basis for the belief that in this work minor contaminations with one cell or the other would not alter the results materially.

With regard to the red cells, a high degree of resistance was produced, and the difference between the results with blood and red cells was not significant statistically. The comparatively large amount of time and handling involved in these experiments seemed to impair the immunizing power of both the blood and the red cells somewhat, but this was not sufficient to alter the important conclusion that a relatively pure suspension of red cells is a potent antigen for the development of induced resistance to tumors. This is not necessarily in conflict with a recent report by Medawar (8) which deals with "immunity" to transplantation of normal tissues. He stated that the prior injection of foreign homologous erythrocytes did not give rise to "appreciable immunity" toward skin from the same donor, in rabbits, but that leukocytes were effective. Although it is generally accepted that the laws governing the transplantation of tumors are the same as those which govern the transplantation of normal tissues, one cannot be sure at the present time that immunity toward normal tissues is in all respects the same as immunity toward tumors, though there are many points of similarity.

In all experiments in which cell suspensions were laked, whether by freezing, by water, or by sound waves, the power to elicit resistance which was present in the control suspension was entirely absent from the test suspension. In no case was anything removed from the test suspension; such residues of cells and cell fragments as may have remained in the test material after treatment were resuspended, and the contents of the test suspension represented the entire contents of the control suspension. Two interpretations are possible; the alteration produced by the treatment of the cells may be primarily chemical or primarily structural. A third interpretation, that inhibitors are released, seems to be ruled out by the experiment of Table 4.

Since almost anything that one might do to a protein or other cellular antigen, however gentle, may be capable of producing a minor degree of chemical change or denaturation, the essentially physical methods used here are each open to some suspicion. However, it is reasonable to believe that such chemical changes as may have occurred in these methods must have been of a minor kind and not the cause of the profound changes in antigenicity observed. The exposure to sonic vibrations seems on the surface to be the most rigorous of the procedures, but the intensity was low, no perceptible heating occurred, and the duration was 2 minutes or less. Hogeboom and Schneider[4] have exposed several enzymes to the same intensity of vibration for very much longer periods of time without observing any evidence of denaturation. One of the strongest arguments against the notion that the change produced was primarily a chemical one is that, though each of the technics may have caused minor chemical changes, it is very difficult to believe that all produced the same change and that this accounts for the results. Denaturation of some special antigen may be the explanation of these results, but, if so, it would seem that the antigen must be labile indeed, and in that case it is difficult to understand how so many of the experiments done in the past succeeded. Still, chemical denaturation of the antigen could have occurred, and that interpretation of this work cannot be ruled out on present information.

If one rejects a chemical interpretation of this work for the reasons just given, there is little or no alternative to an interpretation involving structure.[5] One may invoke such terms as "living" or "devitalized," as many have done in the past, but for the present discussion these factors will be passed over on the grounds that they depend upon conditions that cannot be clearly defined at present. We also prefer to avoid the use of the word "intact" for the time being, because in the present data there is no final basis for choosing between an intact cell and a major portion thereof. On the whole, the results fit well with the working hypothesis that the critical antigenicity or specificity involved in induced resistance is a function of structural organization among the constituent chemical parts, the level of organization lying somewhere above that of complex protein molecules and possibly as high as that of the cell or its surface. Disintegration of such a structure would not necessarily alter the ordinary antigenicity of its constituent parts but would remove its power to elicit the special type of immunity that is known as induced resistance to the implantation of tumors. In that sense the whole seems to be greater than the sum of its parts. The results of these experiments strongly suggest an interpretation based on structural organization, but they are not adequate to prove it. Nevertheless, such

[4] Personal communication from Dr. G. H. Hogeboom.

[5] By "structure" we mean physical organization on a scale approximating that represented by the architecture of a cell. It is recognized that the chemical changes which might be involved may also be "structural," but in that case the scale or level of organization is that of a molecule. Some of the ideas expressed here are reminiscent of those of Landsteiner and Pauling (5), but there, again, the scale of organization is that of the molecule.

an interpretation will form the basis for continuing experiments in this laboratory.

SUMMARY

With mice of strains DBA and C and a sarcoma which arose in strain DBA but is transplantable to both strains, resistance to implantation of the tumor was induced (in strain C) by the prior inoculation of defibrinated blood or fractions thereof. The objective was to confirm and extend previous work and to discover some of the essential attributes of the antigen involved.

It was confirmed that resistance can be elicited by such methods when the host is of a different strain than the tumor but not when the host and tumor are of the same strain.

It was shown that this sarcoma is susceptible to this type of immunity.

The effective antigen was found in the cells and not in the serum, and it was noted further that a relatively pure suspension of washed red cells was an efficient antigen.

The degree of resistance induced was proportionate to the dose of antigen given, both in the case of defibrinated blood and of washed red cells. The minimal dose which can produce a significant degree of resistance was found to be as little as or less than 0.0031 cc. of defibrinated blood or its equivalent in red cells.

The power of a suspension of red cells to elicit resistance was destroyed when the cells were laked by either slow freezing, suspending in distilled water momentarily, or brief exposure to the vibrations of high-pitched audible sound.

The interpretation adopted, tentatively, is that the antigenicity involved is based in some way upon the architectural integrity of the cell, its surface, or a major fraction thereof.

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The Effect of High Levels of Certain Steroids on Induced Lymphocytic Leukemia in the Rat

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If one accepts the established fact that the adrenal steroids have regulatory powers over normal lymphoid tissue, it would seem reasonable to assume that the adrenal steroids may also regulate the immature cells of lymphocytic leukemia.

Since the advent of the clinical use of cortisone and of ACTH in April, 1949, many studies on both animals and human beings have been attempted to determine whether cortisone or the adrenocorticotropic hormone had any effect on the lymphoid cells of neoplastic origin. The results reported in the literature are widely diversified. In some cases of acute leukemia definite improvement, from a hematologic standpoint, was reported. Marrows have been said to revert to a normal picture, and the peripheral blood smears revealed no suggestion of cellular immaturity. These cases, however, have been exceptions. Not only have the great majority of acute leukemias failed to respond, but, at times, the course of the disease has actually appeared to be accelerated by treatment with either cortisone or adrenocorticotropic hormone. Those few remissions that have been reported to occur were never permanent, and, sooner or later, the blood picture changed from the normal back to the characteristic malignant pattern of acute leukemia. Subsequent courses of therapy usually failed to produce any further remissions.

As yet, there has been no satisfactory explanation of the action of the adrenal cortical hormones on the blood elements. However, numerous investigators have concluded that lymphoid tissue, both fixed and circulating, is probably an end organ over which certain steroid hormones of the adrenal cortex have the power of regulation. Evidence for this concept can be found in the earlier medical literature. For example, as early as 1895, Star (10) reported the case of a young girl who had died in an Addisonian crisis; at necropsy, marked enlargement of the thymus and other lymphoid tissues was observed. It was 2 years later that Bardeen (1) reported his observations on patients who had died following extensive superficial burns. These individuals displayed an atrophy or a diminution of the lymphoid tissue. Thus, we might project these two independent observations to our present-day understanding. The low level of adrenal steroids, in Addison's disease, permits or fails to inhibit lymphoid hyperplasia. On the other hand, high levels of circulating adrenal steroids, which are present during the alarm reaction or long periods of stress, are capable of inhibiting normal lymphoid growth. At times involutionary changes will become apparent. The latter situation is analogous to those cases of extensive superficial burns described by Bardeen, when stress was produced, stimulating the release of high levels of steroids into the blood stream.

On the basis of the foregoing evidence it is logical to approach the problem of therapy of leukemia by the administration of adrenal cortical steroids. If the leukemic cell of lymphoid origin has basically the same chemical structure as the immature cell of normal lymphatic tissue, it is conceivable that high levels of adrenal steroid may also inhibit the proliferation of these abnormal cells. Previous studies have failed to establish clearly the hormonal control of these leukemic cells. This suggests either that the leukemic cell does not have the basic physicochemical structure present in the normal cell or that the action of the steroids is blocked by some unknown factor or factors.

Heilman and Kendall (3) induced a regression in the size of growing transplanted lymphosarcomas in mice by the administration of Compound E. While the tumors did show marked regression during the period of therapy, discontinuance of the hormone resulted in regrowth of the tumors with ultimate death of the animal. They concluded that "the influence of Compound E appears to depend upon stimulation of the rate of the catabolism of proteins to a degree which resulted in the death of malignant cells. The normal tissue

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cells of the animal, although they might be placed under severe strain, did not appear to undergo permanent injury."

Prior to the publication of these data, Murphy and Sturm (9) had shown that adrenalectomy significantly reduced the natural resistance of rats to a transplantable lymphatic leukemia. Their percentage of successful transfers was 89.7 per cent in adrenalectomized animals, as compared to 43.5 per cent in a control group of normal animals. Later they showed that an increased resistance to transplantable lymphoid tumors in rats was induced by the administration of adrenal cortical extracts in oil.

Law and Spiers (5) noted a decrease in the total immature lymphocytes in mice with spontaneous lymphatic leukemia after the injection of adrenal cortical extract. Examination of the tissues of these animals revealed a regression in the infiltration of the thymuses and lymph nodes of those animals that received adrenal cortex, as compared to the untreated controls. There was no apparent difference in the mortality rates of the two groups, although the treated group survived for longer periods after the development of leukemia than the controls.

Levin (6) attempted to evaluate the role of the adrenal cortex in lymphoid leukemia. He did this by determining the amount of cholesterol in the adrenal glands of mice before and after the development of the leukemic state. Although the weight of the adrenal glands was increased by 38.0 per cent following the development of leukemia, the amount of cholesterol in these glands was markedly reduced. If we analyze these findings, we can readily see that the leukemic state produced changes in the adrenal gland analogous to the stress produced by lethal doses of toxins, infectious diseases, burns, hemorrhage, and traumatic shock. Thus, there is apparently a nonspecific relationship between the adrenal cortex and the leukemic state, even though the cells of lymphoid origin act as an end organ to the influence exerted by the adrenal cortical steroids.

In attempting to determine the effect of adrenal stimulation on leukemia, Levin administered pituitary adrenocorticotropic hormone from the time of transmission of the leukemia until the time of death in a small series of mice. He was not able to demonstrate any significant difference in the course of the disease in the treated group as compared to the untreated control leukemic animals.

These results with adrenocorticotropic hormone were not in agreement, however, with those of Murphy and Sturm (9). They had shown that ACTH prevented the development of leukemia in 75 per cent of one series of rats, while the control series of animals had a mortality rate of 90 per cent.

Lewis, Aptekman, and King (7) reported a retarding action of the adrenal gland on the growth of sarcoma grafts in rats. By mincing adrenal tissue and implanting it with the tumor fragments they were able to prevent the growth of the tumor in the recipient animal. But if minced kidney, spleen, liver, or thymus tissue were transplanted with the sarcoma, tumor growth was even more rapid than it was in the control group of animals in which the sarcoma had been implanted alone.

Higgins and Woods (4) showed that cortisone did restrict the leukemic process in mice, and they were able to extend the survival time from a mean of 8.7 days to one of 11.9 days. Burchenal, Stock, and Rhoads (2) reported the effects of the administration of both cortisone and ACTH to mice into which AK_4 mouse leukemia had previously been transplanted. They showed that cortisone, and to a lesser degree ACTH, produced an anti-leukemic effect on the normal progress of the disease. This was demonstrated by the absence of, or minimal, leukemic infiltration into the liver of those mice that received cortisone, as compared to the heavy infiltration of cells of leukemic origin into the parenchyma of the control animals. The survival time of the treated animals was not significantly altered from that of the untreated control mice.

METHODS

Rats of the Sprague-Dawley strain, weighing between 60 and 90 gm. and ranging in age from 4 to 6 weeks, were selected for our study. All animals were housed in metal cages, kept on wire screen, and were provided with an adequate commercial ration.

The lymphocytic leukemia was that which had been developed at the Rockefeller Institute by Murphy and Sturm (8). They had produced lymphosarcoma in a Wistar rat by injecting 1,2,5,6-dibenzanthracene subcutaneously into the groin. This tumor was found to be easily transplantable. Unique among its characteristics was its ability to have a dual growth pattern. The subcutaneous injection of a cellular suspension of this tumor produced a rapidly growing lymphosarcoma that killed the rat in a period of from 18 to 22 days. When this material was injected into the peritoneal cavity, an acute fulminating lymphocytic leukemia occurred which produced death in from 7 to 10 days.

At all times an adequate stock of rats with lymphosarcoma was kept on hand, from which donor tissue could be obtained. These lymphosarcomas

grew to be very large, often attaining a size of 7×4 cm. by the third week. They rarely metastasized to distant parts of the body, but they invaded the neighboring tissues by direct extension. Despite the large size of these tumors, central necrosis rarely occurred. This was most unusual, since there is a relative avascularity, as would be expected in any tumor with so fast a growth. The tumor is unusually firm, and the cut surface is glistening and white. The lymphosarcoma is particularly suitable for transplanting to large numbers of animals, since most of the cells are viable and no tissue has to be discarded. Histologically, the tumor is composed of small round cells, which are uniform in size. Scattered throughout are larger cells with definite reticular characteristics. In the young tumor, many mitotic figures may be seen. The stroma is scant in the small tumors but increases in amount with the age and the size of the lymphosarcoma.

We have found that it is possible to control the degree of malignancy by varying the age of the donor cells. Thus, we can increase the malignancy and lower the life expectancy in the second generation by allowing the donor tissue to achieve a maximal period of growth. Conversely, the life expectancy can be prolonged in the second generation by utilizing the donor tissue after a short period of growth.

Transfers were made of donor tissue, obtained by a sterile method, from animals into which tumor tissue had been implanted 9–11 days previously. A cellular suspension was prepared in the following manner: the lymphosarcoma was removed from donor animals immediately after they had been killed by etherization. Sterile iridectomy scissors and small thumb forceps were used in cutting the tumor into small fragments, which were then macerated in a sterile crucible. A double volume of sterile isotonic saline solution was then added to the tissue, and the resultant mixture was strained through four layers of gauze. One-half cc. of this cellular suspension was injected into the peritoneal cavity of each animal.

Blood samples were obtained from tail veins, and total leukocyte and differential distributions were determined in the usual way. Adrenocorticotropic hormone and cortisone were given intramuscularly every 12 hours during test periods.

RESULTS

Induced leukemia in the normal animal.—Six series of animals, with a total of 240 rats, were used in evaluating the total leukemic picture in this strain of rats. All these animals were approximately the same size and weight. The percentage of successful transplants resulting in a fulminating leukemia in the six series of normal animals ranged from 84 to 100 per cent, with an over-all take of 88 per cent. That is, 211 of the 240 animals inoculated with 0.5 cc. of the cellular suspension died with acute lymphocytic leukemia. The blood of 60 of these leukemic animals was examined daily to appraise the changes in the total and in the differential leukocyte counts. Not all the animals developed leukocytosis, for some of them developed marked leukopenia.

Necropsy was performed on all 60 animals, and sections of the adrenals, thymus, spleen, liver, and lymph nodes were prepared (Figs. 1 and 2). In every animal leukemic infiltration into the thymus, the lymph nodes, mesentery, liver, spleen, and bone marrow was noted. The most striking changes were observed in the thymus, where the leukemic infiltration produced as much as a 400 per cent increase in size. The leukemic infiltration into the marrow completely overshadowed the myelopoiesis or erythropoiesis which is normally present. The leukemic cell infiltration into the liver was concentrated mainly around the portal vessels at the periphery of the lobules, but there was some dissemination around adjacent sinusoids. There was no apparent infiltration into the adrenal glands, and yet the weights of these organs were considerably increased when compared to the weights of adrenals of those few animals in which leukemia had failed to develop (Chart 1).

The effect of ACTH in the normal animal.—Ten normal, male rats, varying in weight between 65 and 80 gm., were given intramuscularly 1 mg. of ACTH per rat every 12 hours for a period of 10 days. These animals were examined every third day to determine changes in body weight as well as changes in the total and the differential leukocyte counts. On the eleventh day, necropsy was performed on five of the ten animals, and five were allowed to live for 5 days longer.

A decrease in the total number of leukocytes was observed in each animal that had received ACTH, with a relative and absolute decrease in the number of lymphocytes and a relative and absolute increase in the number of neutrophilic leukocytes. After the withdrawal of ACTH, there was some tendency for the number of lymphocytes to return to normal, but the initial level had not been attained at the time the animals were killed on the sixteenth day. The total number of neutrophils quickly dropped to the initial level 2 days after ACTH was discontinued.

In animals, killed on the eleventh day, that had received ACTH for the preceding 10 consecutive days, a significant increase in the size of the adre-

nal glands and a marked decrease in the size of the thymuses were noted when compared to the weights of these organs in untreated controls of the same age and body weight.

Discontinuance of the ACTH for 5 days resulted in a significant gain in body weight. The thymus showed some tendency to regenerate during the 5-day recovery period, and the adrenals were smaller than before, approaching the size of the adrenals of the untreated controls.

The effect of cortisone in the normal animal.—Cortisone was administered intramuscularly to 10 rats for 10 days in amounts of 1 mg. every 12 hours. Five animals were killed on the eleventh

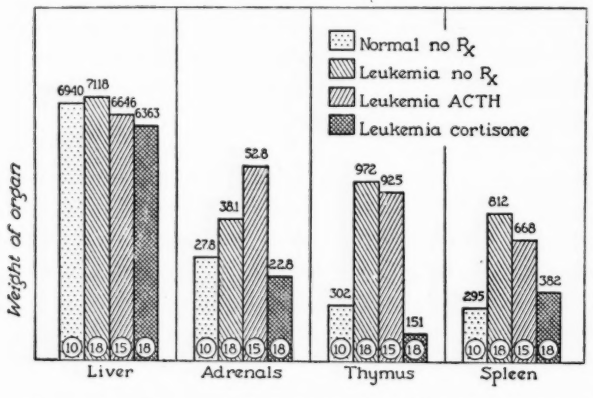

CHART 1.—Organ weights of animals with leukemia revealing deviation from normal, as well as changes provoked by ACTH and cortisone therapy. All weights are expressed in milligrams per hundred grams of body weight. Number of animals on which necropsy was performed is given in circles.

day, and the remaining animals were killed on the sixteenth day, 6 days after cortisone was discontinued.

The total leukocyte count in all rats receiving cortisone dropped to a much lower level with a greater relative and absolute decrease in the lymphocytes than occurred in animals receiving ACTH. The relative and absolute neutrophilia was also more marked in rats on the cortisone regimen than in those given ACTH.

The animals appeared lethargic on the eighth day of the administration of cortisone. The lethargy, however, was not progressive, and their activities were only moderately less than normal on the last day of the administration of the hormone. Loss of body weight was about the same in each animal during this 10-day period. Discontinuance of the cortisone resulted in a significant gain in weight in the five rats that were allowed to recover from the toxic effects of such large doses of hormone. There was marked thymic atrophy in animals receiving cortisone, and their adrenals were atrophic, as was anticipated.

On withdrawal of cortisone from five of the animals on the tenth day, a prompt improvement in activity and an increase in body weight were recorded. At necropsy, 6 days later, the thymus and the adrenals were significantly larger than those observed in animals killed after 10 days of cortisone therapy.

The effect of ACTH in animals bearing a transplanted lymphatic leukemia.—Forty rats of approximately the same size were inoculated with leukemia in the manner described previously. On the fourth day after inoculation of the leukemic cells, ACTH therapy was instituted on 20 of these 40 animals in amounts of 1 mg. every 12 hours. Intramuscular injections were continued for 10 days to all animals that survived for this entire period of treatment. The approximate time of death was recorded for each animal comprising both the test and the control groups.

The results of this initial study with adrenocorticotropic hormone indicated that its administration to rats bearing leukemia had ameliorated the disease process somewhat. There was an increase in the survival time of the animals that received ACTH; but the difference in the mortality rate in the two groups was so slight as to be statistically insignificant, for a 20 per cent survival was recorded for the group which received ACTH, while 10 per cent of the untreated control group survived (Chart 2).

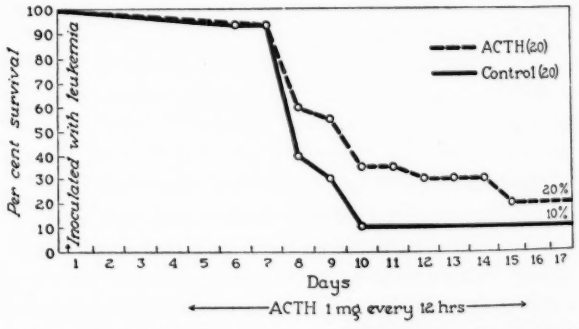

CHART 2.—Viability curve. ACTH begun after leukemia had been established.

Since leukemia in these animals had developed for 4 days and was certainly fulminating, it was thought that the proliferation of leukemic cells had reached such a magnitude at the time ACTH was first given as to make the treatment relatively ineffective.

Accordingly, a second series of 45 animals of the same size and age was selected to constitute a second test group. Twenty-five of these served as controls, and 20 were inoculated with leukemic cells. On the day of inoculation and daily thereafter these 20 rats were given 1.0 mg. of ACTH

every 12 hours for a period of 12 days. The blood of each animal in the hormone-treated group was studied on the first, fourth, and eighth day of therapy to determine the effect of ACTH on the leukocyte distribution.

There was no appreciable change in the total leukocyte count of the treated animals when they were compared to the animals that had received no treatment. But there was a marked neutrophilia, with an average of about 20,000 neutrophils per cubic millimeter of blood, recorded on the eighth day for animals given ACTH. The number of lymphocytes, both mature and immature, was considerably reduced, but again there was no appreciable difference in the mortality rates of the treated and the untreated groups. Survival time was only slightly prolonged: those leukemic animals receiving ACTH from the day they were inoculated lived, on the average, only 24 hours longer than the untreated controls (Chart 3).

Weight changes of the organs in the ACTH-treated group varied but slightly from those in the control group (Chart 1). Decreases in the weights of the spleen and thymus were due primarily to the lysis of normal lymphoid tissue induced by the hormone, and the marked increase in weight of the adrenal glands was, of course, due to its corticotropic effect. A slight decrease in the weight of the liver represented, we believe, an actual retardation of the leukemic infiltration into this organ.

Histologic examination of certain tissues showed marked changes produced by ACTH. The malignant invasive pattern, however, characteristic of control leukemic animals was maintained in many organs of those receiving ACTH; but there were alterations in the cells of leukemic origin. There were marked degenerative changes in these infiltrating cells, consisting of a decrease in their size and a marked vacuolization of their cytoplasm. In all lymphoid tissues throughout the organism a lympholysis of all normal cells of lymphoid origin had occurred. Normal lymphocytes were not seen in any of the sections of the thymus that we examined (Fig. 2*b*). There was a definite decrease in the extent of leukemic infiltration into both the liver (Fig. 1*b*) and the spleen of the ACTH-treated animals. In a few test animals the leukemic infiltration was minimal, but in every instance there was some involvement of the pertinent organs. The bone marrow in ACTH-treated animals showed little if any change from the pattern of extensive leukemic involvement seen in the marrows of the control leukemic animals.

The effect of cortisone on transplanted lymphatic leukemia.—Twenty animals of the same sex and age as used in the ACTH study were selected and inoculated with leukemic cell suspension. One mg. of cortisone was administered every 12 hours for a period of 12 days to all animals that survived for this period of time. Cortisone was given on the day of the transplant and on each succeeding survival day for 12 days.

Blood of these animals was examined on the first, fourth, and eighth day following inoculation for the changes in the total and the differential leukocyte distribution. Counts taken after the eighth day were considered statistically unreliable, as the viability rate decreased precipitously beyond this time, making it virtually impossible to record accurate data on so small a number of animals.

The administration of cortisone produced a

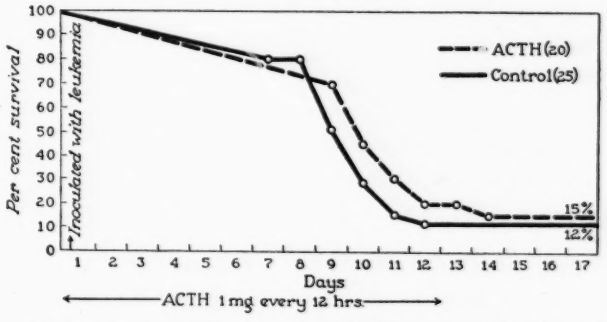

CHART 3.—Viability curve. ACTH begun at the time of the leukemic transplant.

rather marked leukocyte response in these leukemic animals. There was a marked lymphopenia, followed by a relative and an absolute increase in the number of neutrophils. The total number of neutrophils in these leukemic animals, however, never attained so high a level as it did in normal animals given the same amounts of cortisone. This was considered caused by the fact that in leukemic animals most of the myelopoietic centers had been reduced or destroyed by the leukemic infiltration.

The average weight of the thymus in leukemic animals receiving cortisone was less than one-sixth that of the thymus in leukemic animals which did not receive the hormone (Chart 1). Histologically, the thymus consisted essentially of circling strands of loose stroma, separated by a few undifferentiated cells (Fig. 2*c*). True lymphocytes were not encountered in the thymus nor in any other organ. Approximately the same destructive effect of these amounts of cortisone was observed in the spleen as in the thymus, but to a lesser degree. Leukemic infiltration into the liver had not occurred, and the perivascular spaces were entirely free of leukemic cells; only normal parenchymal cells of the liver were present (Fig. 1*c*).

An interesting sidelight to this study was noted

on examination of histologic sections of the pituitary gland. So-called Crooke's changes, which consist of a hyalinization and a vacuolization of the basophil cells, were present in all the pituitary glands of animals which had been given cortisone. These changes in the basophils, which are believed to be induced by high levels of circulating steroids, certainly gave evidence of the intensity of the therapy we used in these leukemic animals.

Despite the apparent lytic effect of cortisone on the leukemic infiltration in the liver and the histology of the spleen and thymus, significant differences in the mortality rates of control and test groups of animals did not occur (Chart 4). Again, however, and to a much greater degree than in leukemic animals receiving ACTH, the survival time of these animals receiving cortisone was prolonged. The cortisone-treated animals lived for an average of 12 days, as compared to an average of 9 days for the untreated leukemic animals.

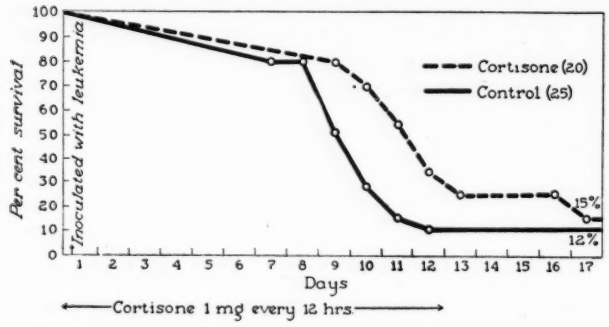

CHART 4.—Viability curve. Cortisone begun at the time of the leukemic transplant.

CONCLUSIONS

A study designed to appraise the influence exerted by either adrenocorticotropic hormone or cortisone on the course of a transplanted lymphatic leukemia in rats has been reported. The data appear to warrant the following conclusions:

1. High levels of either ACTH or of cortisone—1 mg. every 12 hours during the survival period, following inoculation—did exert a suppressing influence on the neoplastic lymphoid tissue. These hormones did not prevent the development of the leukemia, following intraperitoneal inoculation with leukemic cells, but each appeared to ameliorate the disease process somewhat and to prolong the survival time slightly.

2. High levels of these hormones definitely altered the histologic structure of the thymus, the spleen, and other lymphoid organs. Leukemic infiltration into the liver was definitely restricted by the administration of these hormones.

3. Leukemia, thus induced, exerts a severe stress phenomenon within the animal, resulting in marked hypertrophy of the adrenal cortices and an elevation of the absolute numbers of neutrophilic leukocytes in the peripheral blood.

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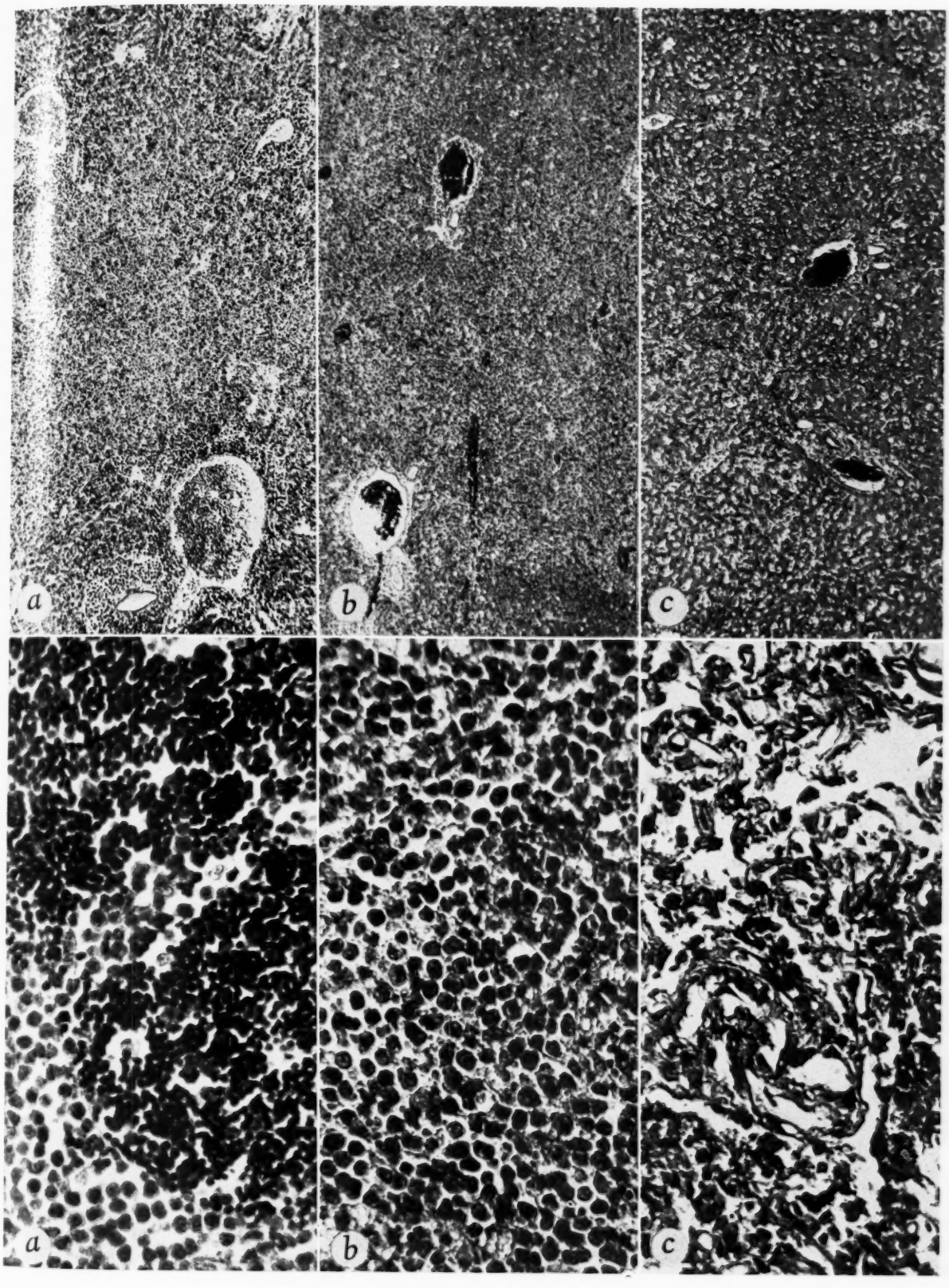
FIG. 1 (*top three figures*).—*a:* Liver, control leukemia. *b:* Liver, ACTH for 10 days. *c:* Liver, cortisone for 10 days. All three animals died with leukemia (hematoxylin and eosin, ×60).

FIG. 2 (*bottom three figures*).—*a:* Thymus. Control leukemia showing the mass of leukemic cells wedging into an area of the thymic cortex (hematoxylin and eosin, ×400). *b:* Thymus. All normal lymphoid tissue has been replaced by malignant cells which are all undergoing degenerative changes. This rat received ACTH for 10 days (hematoxylin and eosin, ×400). *c:* Only a few undifferentiated cells are all that remain of this thymus following 10 days of administration of cortisone. This animal died of leukemia on the eleventh day of the disease (hematoxylin and eosin, ×400).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Organ Erythrocyte and Plasma Volumes of Tumor-bearing Mice

The Oligemia of Neoplasms*

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It was noted a few years ago that transplantable granulosa tumors are associated with marked cavernous congestion of certain viscera concomitant with hypervolemia (1). The pathogenesis of this hypervolemia is unknown. It was found that there is a direct relation between the degree of congestion and the rise in blood volume and that in these hypervolemic animals the albumin concentration in blood is maintained while the globulin concentration tends to drop (11). One of the working hypotheses concerning the nature of this hypervolemia postulates a primary stimulation of albumin production, another a primary cavernous dilatation of certain capillaries. Both call for the determination of organ volumes in order to find out if this process is initiated in the tumor or in an organ. The answer to this problem is still forthcoming, but the accidental discovery made in pursuit of this problem that the tumors contain very little blood and the novel data on cell and plasma volumes and hematocrit values of different organs in both normal and tumor-bearing mice prompted release of our data.

EXPERIMENTAL

Transplantable tumors.—The strains used and the methods of transplantation have been described (3). The recipient mice were animals of the same strain in which the tumor arose.

Red cell, plasma, and organ volumes.—Red cell volumes were determined by the injection of homologous erythrocytes labeled with P^{32} according to the method of Hevesy and Zerahn (6) as modified by Nieset *et al.* (9). Plasma volumes were determined with homologous plasma iodinated with I^{131} by the procedure of Fine and Seligman (2) with a modification by Wish *et al.* (15).

Organ volumes were determined as follows: The mice were anesthetized with an intraperitoneal injection of nembutal; then 0.1–0.2 ml. of either labeled cells or plasma was injected into the tail vein. Five to 6 minutes after injection, blood was withdrawn from the heart and the animal sacrificed with ether. The hematocrit of the heart blood was determined by the capillary technic of Parpart and Ballentine (10). The animal was then wrapped in aluminum foil and placed in an oven at 70° C. for 30 minutes to coagulate the blood; the organs were then excised.

Radioactivity measurements.—For I^{131} determinations, each organ was placed in a tared lusteroid tube for weighing, and the activity was then determined by inserting the tube containing the organ into the gamma-ionization chamber (12). The total activity injected into the animal was determined by lowering the mouse into the gamma-chamber. The I^{131} activity of plasma was similarly determined in the gamma-chamber. In order to detect a poor injection, the tail of each mouse was removed and its activity determined.

P^{32} activity was measured with an autoscaler equipped with automatic sample changer (Tracerlab Co., Boston, Mass.). The weighed organ was dissolved in hot concentrated nitric acid with the aid of 30 per cent hydrogen peroxide and the resulting solution diluted to a known volume. An aliquot was then evaporated to dryness in a metal cap and counted in the autoscaler. Significant self-absorption was avoided by keeping the total solid content low and of a similar order of magnitude for each sample. Activity of the solution used for injection and of the heart blood was determined in the autoscaler after appropriate dilution, adjustment of solid content, and drying.

Calculation of the plasma and cell volume was

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† With the assistance of E. J. Beale and M. M. Knoohuizen.

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made in the usual manner on the basis of the dilution of the injected activity. Since the activity of plasma was measured in the I^{131} determinations, the hematocrit was not used for calculation except for estimation of total blood volume according to the scheme previously reported (15). P^{32} determinations were made on whole blood and corrected to activity per milliliter of cells on the basis of hematocrit values obtained on the same blood sample.

RESULTS

The results of cell and plasma volumes, large vessel and average body hematocrits on all mice used in this study are summarized in Table 1. The cell and plasma volumes of the tumors and four organs determined are shown in Table 2.

TABLE 1

CELL, PLASMA, AND TOTAL BLOOD VOLUMES AND HEMATOCRITS OF ALL ANIMALS STUDIED

Strain	Sex	No. of determinations: Direct plasma	Direct cell	Total	Plasma: Per cent weight	Plasma: Per cent ± from normal	Cell: Per cent weight	Cell: Per cent ± from normal	Total: Per cent weight	Total: Per cent ± from normal	Hematocrit: Peripheral	Hematocrit: Av. body	Hematocrit: Av. body peripheral
Normal	♀	4	5	9	4.9		3.5		8.4		43.4	41.7	0.96
	♂	12	6	18	5.1		2.7		7.8		42.4	34.6	0.82
Granulosa	♀	4	3	7	11.3	+131	3.4	− 2.9	14.7	+ 75	27.2	23.1	0.85
	♂	7	5	12	9.1	+ 78	2.5	− 7.4	11.6	+ 49	30.0	21.6	0.72
Ovarian Ca.	♂	4	1	5	7.9	+ 55	2.0	− 26	9.9	+ 27	26.9	20.2	0.75
Luteoma	♀	1		1	5.4	+ 10.2	3.6	+ 2.9	9.0	+ 7.1	46.2	40.0	0.87
	♂		3	3	11.7	+129	5.2	+93	16.9	+117	42.4	30.8	0.73
Breast Ca.	♀	4	4	8	8.5	+ 74	2.6	− 4.4	11.1	+ 31	26.0	23.4	0.90
	♂	3		3	8.0	+ 57	2.7	0	10.7	+ 37	29.1	25.2	0.87

The data in Table 1 indicate that the total blood and plasma volumes of tumor-bearing mice are greater than those of normal mice. The unique luteoma, which is associated with masculinization and polycythemia (5), has an increased erythrocyte volume, whereas the other tumor strains studied have a decreased cell mass. This overcompensation with plasma for loss of erythrocyte mass seems to be a universal phenomenon (3).

The organ blood volumes and the oligemia of tumors, the most striking finding of this study, are shown in Table 2. Whereas normal livers have blood volumes of 29 per cent of organ weight, lungs 39.6 per cent, and kidneys 25 per cent, the tumors studied contained only 1.5 to 4.7 per cent of blood. Livers, kidneys, and lungs of the tumor-bearing mice studied had higher blood volumes than did those of normals, with the exception of livers of mice bearing breast carcinomas. The spleens of tumor-bearing mice, on the contrary, had a lower blood volume than those of normal mice.

It is possible that tumors have a sluggish circulation. However, extending the mixing time of the injected labeled material from the conventional 6 to 30 minutes still yielded low values for the blood volume of tumors. A total of four normal mice, six mice bearing granulosa tumor, and four mice bearing breast carcinoma were tested in this manner. The iodinated plasma volume per gram of tissue increased during this time in liver (+29 per cent) and kidney (+8 per cent), decreased in lung (−7 per cent), and remained the same or was increased very slightly in tumor. During this period of 30 minutes, some redistribution of the injected protein between blood plasma and lymph had occurred. There are no data available at present to indicate how rapidly equilibration of plasma between blood and lymph spaces takes place in mice, and it is therefore not possible to give a precise interpretation of the above observations. The gain in radioactivity of liver and kidney may be due not to congestion or mixing but to the abundant supply of lymphatics and the lack of gain in activity by tumors to a negligible lymphatic circulation. In any event, the low activity of tumors is not due to sluggish circulation. Since it is now possible to estimate the lymph space of organs,[1] the congestive changes in organs and lymph volume could be quantitated.

It is now well known that the average body hematocrit of dogs is lower than the large vessel hematocrits and that each organ of dogs has a characteristic hematocrit (4). A comparison of the data in dogs (4) with those in mice (Table 2) indicates that the spleen has the highest hematocrit in both species. In normal mice, 68 per cent of the blood volume of the spleen is due to erythrocytes. In three strains of tumor-bearing animals, this

[1] R. H. Storey, J. Moshman, and J. Furth. A Simple Procedure for Determination of the Approximate Lymph Space (to be published).

TABLE 2

Organ and Tumor Blood Volumes of Normal and Tumor-bearing Mice

Organ	Tumor strain	No. of tests: Plasma volume	No. of tests: Cell volume	Plasma volume: Per cent wt.	Cell volume: Per cent wt.	Total blood volume: Per cent wt.	Parenchyma: Per cent wt.	Organ hct.	Organ hct. / Av. body hct.	Weight: Per cent body	Weight: Per cent body minus tumor
					I. Females						
Tumor	Granulosa	4	3	4	0.9	4.9	95.1	18.4	0.80	22.1	
	Luteoma	1		2.0						26.1	
	Breast ca.	3	4	4.0	0.7	4.7	95.3	14.9	0.64	29.2	
Liver	Normal	5	4	18.0	13.0	31.0	69.0	42.0	1.01	6.2	
	Granulosa	4	3	26.0	9.0	35.0	65.0	25.7	1.11	7.7	9.9
	Luteoma	1		23.0						7.2	9.8
	Breast ca.	4	4	14.0	4.0	18.0	82.0	22.2	0.95	7.8	11.4
Spleen	Normal	3	4	8.0	22.0	30.0	70.0	73.5	1.76	0.8	
	Granulosa	1	3	11.0	8.0	19.0	81.0	42.1	1.82	0.87	1.12
	Luteoma	1		6.0						0.91	1.2
	Breast ca.	1	4	14.0	13.0	27.0	73.0	48.2	2.06	0.75	1.08
Kidney	Normal	4	4	16.0	9.0	25.0	75.0	36.0	0.86	1.3	
	Granulosa	4	3	34.0	9.0	43.0	57.0	20.9	0.91	1.14	1.45
	Luteoma	1		19.0						1.4	1.9
	Breast ca.	3	4	19.0	5.5	24.5	75.5	22.4	0.96	1.05	1.43
Lung	Normal	3	2	17.0	16.0	33.0	67.0	48.5	1.16	0.72	
	Granulosa	2	2	31.0	15.0	46.0	54.0	32.6	1.41	0.68	0.84
	Luteoma	1		24.0						0.56	0.77
	Breast ca.	1		39.0						0.71	0.97
					II. Males						
Tumor	Granulosa	7	2	3.0	0.9	3.9	96.1	23.1	1.07	18.4	
	Ovarian ca.	4	1	1.0	0.5	1.5	98.5	33.3	1.65	33.6	
	Luteoma		3		1.5					22.2	
	Breast ca.	3		4.0						24.9	
Liver	Normal	13	3	15.0	12.0	27.0	73.0	44.5	1.28	6.0	
	Granulosa	7	2	29.0	6.0	35.0	65.0	17.2	0.79	8.0	9.7
	Ovarian ca.	4	1	30.0	6.0	36.0	64.0	16.7	0.83	5.7	8.7
	Luteoma		3		11.0					9.1	12.5
	Breast ca.	3		19.0						7.5	10.2
Spleen	Normal	10	2	12.0	27.0	39.0	61.0	69.2	2.0	0.5	
	Granulosa	6	4	13.0	3.0	16.0	84.0	18.8	0.87	0.85	1.02
	Ovarian ca.	3	1	10.0	3.0	13.0	87.0	23.1	1.15	0.77	1.2
	Luteoma		3		12.0					0.58	0.7
	Breast ca.	1		19.0						0.67	1.15
Kidney	Normal	12	3	14.0	10.0	24.0	76.0	41.7	1.20	1.9	
	Granulosa	7	2	23.0	5.0	28.0	72.0	17.9	0.83	1.48	1.89
	Ovarian ca.	3	1	26.0	5.0	31.0	69.0	16.1	0.80	1.2	1.7
	Luteoma		3		11.0					1.5	1.9
	Breast ca.	2		25.0						0.85	1.28
Lung	Normal	7	1	25.0	19.0	44.0	56.0	43.2	1.25	0.77	
	Granulosa	4	1	27.0	16.0	43.0	57.0	37.2	1.72	0.70	0.79
	Ovarian ca.		3		27.0					0.55	0.84
	Luteoma		1		23.0					0.55	0.74
					III. Weighted averages of both sexes						
Tumor	Granulosa	11	5	3.4	0.9	4.3	95.7	20.9	0.95	20.1	
	Ovarian ca.	4	1	1.0	0.5	1.5	98.5	33.3	1.65	33.6	
	Luteoma	1	3	2.0	1.5	3.5	96.5	42.8	1.29	23.2	
	Breast ca.	6	4	4.0	0.7	4.7	95.3	14.9	0.62	27.9	
Liver	Normal	18	7	15.8	12.6	28.4	71.6	44.4	1.20	6.1	
	Granulosa	11	5	27.9	7.8	35.7	64.3	21.8	0.99	7.9	9.8
	Ovarian ca.	4	1	30.0	6.0	36.0	64.0	16.7	0.83	5.7	8.7
	Luteoma	1	3	23.0	11.0	34.0	66.0	32.4	0.98	8.6	11.8
	Breast ca.	7	4	16.2	4.0	20.2	79.8	19.8	0.83	7.7	11.1
Spleen	Normal	13	6	11.1	23.7	34.8	65.2	68.1	1.84	0.6	
	Granulosa	7	7	12.7	5.2	17.9	82.1	29.1	1.32	0.86	1.05
	Ovarian ca.	3	1	10.0	3.0	13.0	87.0	23.1	1.15	0.77	1.2
	Luteoma	1	3	6.0	12.0	18.0	82.0	66.7	2.02	0.66	0.83
	Breast ca.	2	4	16.5	13.0	29.5	70.5	44.1	1.85	0.74	1.09
Kidney	Normal	16	7	14.5	9.4	23.9	76.1	39.4	1.07	1.69	
	Granulosa	11	5	27.0	7.4	34.4	65.6	21.5	0.97	1.33	1.7
	Ovarian ca.	3	1	26.0	5.0	31.0	69.0	16.1	0.80	1.2	1.7
	Luteoma	1	3	19.0	11.0	30.0	70.0	36.6	1.11	1.48	1.9
	Breast ca.	5	4	21.4	5.5	26.9	73.1	20.4	0.85	1.01	1.4
Lung	Normal	10	3	22.6	17.0	39.6	60.4	43.0	1.16	0.75	
	Granulosa	6	3	28.3	15.3	43.6	56.4	35.1	1.59	0.69	0.81
	Ovarian ca.		3		27.0					0.55	0.84
	Luteoma	1	1	24.0	23.0	47.0	53.0	48.9	1.48	0.56	0.76
	Breast ca.	1		39.0						0.71	0.97

value is 23.1–44.1 per cent; in mice bearing luteomas it is 66.7 per cent. The latter tumor, it should be recalled, causes polycythemia (5).

The liver hematocrits of normal mice are similar to those of large vessels, averaging 44.4 per cent. The same is true for dogs (4). The liver hematocrits of tumor-bearing mice are below the average body hematocrit. In mice bearing tumors other than luteomas, the liver hematocrits varied between 16.7 per cent and 21.8 per cent, while in animals with luteoma they averaged 32.4 per cent. The organ hematocrits of the lung of normal and granulosa tumor-bearing mice of both sexes were higher than the average body hematocrit.

The data of Table 2 indicate that the erythrocyte-plasma ratio differs not only from organ to organ but also from neoplasm to neoplasm.

Kidney weight is known to be under the influence of gonadal hormones. In our series, the weight of kidneys of normal female mice was 1.3 per cent of the body weight and that of males was 1.9 per cent. The masculinizing luteoma raised the kidney weight of females to 1.9 per cent of body weight. In luteoma-bearing males, the kidney weight was not above normal, but the erythrocyte mass was above normal. In granulosa tumor-bearing mice, the weight of kidneys of females was 1.5 per cent of body weight, and in males the corresponding value was 1.9 per cent. There was no sex difference in the volume of blood in kidneys of normal mice, but in granulosa tumor-bearing females the blood volume of this organ was 43 per cent, while in males it was only 28 per cent. It should be recalled that hypervolemia is a characteristic feature of transplanted estrogen-producing tumors.

DISCUSSION

Technics of organ volume determination.—In the course of our studies on blood and organ volume, certain technical errors have become clear which also apply to the related work of others. The use of heterologous protein for determination of plasma volume may lead to false values. To avoid this, homologous blood protein and preferably albumin is utilized since globulin may carry isoantibodies. Sampling after a "mixing time" of 5–6 minutes has been found by us to be essential; after this time, there is a drop which is particularly marked when heterologous protein or cells are used (14). Prolonging this mixing time to 1 hour, as has been reported, yields false values. During this period some of the injected protein is metabolized, and a considerable amount of it enters the lymph space. Studies by Krieger *et al.* (8), Wasserman and Mayerson (13), and in our laboratory[2] on dogs show that 1 hour after injection of labeled protein, thoracic lymph contains 5–20 per cent as much activity per milliliter as does the plasma; almost no activity is present in the lymph 5–6 minutes after injection. Krieger *et al.* found this 1-hour value to be much greater when heterologous protein was used, whereas Wasserman and Mayerson observed no such difference. In experiments with heterologous protein, Krieger *et al.* used manual pressure on the abdomen to increase the lymph flow and state that it is possible that this produced trauma with increased permeability. In our experiments (14), there was a difference between the rate of loss of homologous and heterologous proteins from the circulation, particularly after a prolonged mixing time. In our laboratory, the plasma volume of 27 normal mice averaged 5.0 per cent of body weight when homologous protein and mixing times of 5–6 minutes were used; with heterologous protein and mixing times of 15–60 minutes, Kaliss and Pressman (7) obtained average values of 6.7 per cent of body weight in nine normal mice.

The micro-hematocrit technic of Parpart and Ballentine (10) was found to be satisfactory, and through its use it was possible to determine the hematocrit on each mouse. When calculating total blood volumes from either cell or plasma volumes, the peripheral hematocrit must be converted to the average body hematocrit, which in mice was found to be 0.88 times the large vessel hematocrit (15). Hematocrits of blood from the tail vein are significantly lower than those of heart blood.

A weakness in our technic was the removal of relatively large amounts of blood from the heart of the nearly dead mouse for the measurement of plasma volume. The sample removed (approximately 0.3 ml.) may amount to as much as 15–20 per cent of the total blood volume. If no redistribution of plasma was caused by removal of this blood, the relative values should be in the proper relation; otherwise, the average organ plasma volumes may be low by a factor of about 10 per cent. This difficulty could be overcome by the use of tagged albumin with higher specific activity and the use of a more sensitive counter. The problem of agonal redistribution of blood will require special study.

Organ cell volume determinations are not subject to the above criticism, since the quantity of

[2] R. H. Storey, J. Furth, M. C. Woods, and R. R. Bigelow. Lymph Volume, Clearance and Metabolism Rates of Introduced Labeled Albumin in Normal and Irradiated Dogs (in preparation).

blood withdrawn for P^{32} activity determinations was less than 5 per cent of the total blood volume.

A simpler cell volume determination than here reported could be made with Fe^{59}-tagged erythrocytes with high specific activity. Fe^{59} has high-energy gamma rays and can therefore be accurately measured in the gamma-chamber (12). Thus, weighing the organ and measuring its radioactivity would suffice to determine the organ cell volume.

Blood volume and hematocrit of organs.—Gibson *et al.* (4), in a thorough study, determined the distribution of cells and plasma in the large and minute vessels of the dog and the hematocrit of small vessels of various organs. In our study, the entire organ was measured, thus including blood in both large and minute vessels. Data on the mouse reported by Kaliss and Pressman (7) were obtained by the use of I^{131}-labeled heterologous protein. They calculated the total blood volume on the basis of peripheral and not of average body hematocrits. The following is a comparison of these values for plasma volume in ml/100 gm.

	Mice		Dog
	Kaliss and Pressman	Present Study	Gibson
Whole animal	6.7	5.0	
Kidneys	19.1	14.0	17.4
Liver	20.2	16.5	11.5
Lungs	27.4	21.0	11.5
Spleen	9.2	10.0	6.5

The data here presented of direct erythrocyte and plasma volumes in organs of normal and tumor-bearing mice are novel. In many respects they are sketchy. Refinements of technic and amplifications such as relation of organ volume to tumor size and severity of anemia, the use of tumor strains of different type, and assay of different organs with a material balance are desirable.

SUMMARY

Direct determinations were made of the cell and plasma volumes of organs and tumors; on this basis, the organ and average body hematocrits were calculated. Each organ has a characteristic hematocrit with values diminishing in the following order: spleen, liver and lung, kidney.

The blood volume of all tumors examined was much below that of these organs. Experiments indicate that this oligemia of tumors is real and not due to sluggish circulation.

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Tocopherol in Tumor Tissues and Effects of Tocopherol on the Development of Liver Tumors*

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Most of the attempts made to relate vitamin E to cancer have dealt with possible effects of the vitamin on tumor formation rather than with the metabolism of vitamin E in tumor tissue. Tocopherol has occasionally been observed to delay the development of tumors due to hydrocarbons (3, 8, 9, 34), but this effect has not been consistent (5, 16), nor does vitamin E seem to affect the growth of implanted tumors (10, 22, 43). At one time wheat germ oil was considered carcinogenic (31, 32), but attempts to repeat the original experiments failed (4, 17, 32). Vitamin E has been shown to be important in the prevention of certain liver disorders of dietary origin (18, 33), including massive hepatic necrosis caused by feeding the azo dye, *p*-dimethylaminoazobenzene (DAB) (15). If these results were applicable to liver tumors due to azo dyes, one might expect vitamin E to delay the formation of this latter type of tumor. However, no such effect was observed when rats fed DAB also received tocopherol in the relatively moderate amounts necessary to equalize the tocopherol intake of rats fed hydrogenated coconut oil or corn oil (23).

As far as we are aware, only one study has been made of the amounts of vitamin E in tumor tissue. V. Euler and v. Euler (38) reported that the Jensen sarcoma in the rat and the Brown-Pearce sarcoma in the rabbit contained 50 μg/gm and 44 μg of tocopherol/gm, respectively. In a later report (39), these workers found only 9 μg of tocopherol/gm of tumor, an amount considered similar to that in normal tissues rich in vitamin E. The present study involves two approaches to the problem. In the first, analyses were made of the tocopherol content of tumors of rats fed various levels of tocopherol or that had been deprived of tocopherol for various periods of time. In the second, tumors were produced in rats by feeding 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) in diets high or low in tocopherol, or diets were fed that presumably increase the need for the vitamin.

METHODS

Rats bearing hepatomas due to 3′-Me-DAB were placed in wire-bottom cages and given food and water ad libitum. The animals were maintained either on basal diet I composed of extracted casein, 12; glucose monohydrate (Cerelose), 79; corn oil, 5; and salts, 4, with vitamins added at the following levels in mg/kg of diet: thiamine, 3; riboflavin, 2; pyridoxine, 2.5; calcium pantothenate, 7.5; and choline chloride, 1,000; or on diet II which contained an additional 1–1.6 mg of α-tocopherol/gm. Depletion was studied by placing rats previously fed the diet high in vitamin E on diet I for 8–14 days.

Other rats received multiple subcutaneous inoculations of minces of the Flexner-Jobling carcinoma or of small pieces of Walker carcinoma, or a mince of the Jensen sarcoma was introduced intramuscularly.[1] Ten rats received each type of tumor. These were distributed randomly into two groups, one of which received the basal ration I, while the other received diet II for 10–20 days until the tumors were 1–10 gm. in weight.

The rats were killed by decapitation, and the tumor tissue was excised and freed as much as possible from adjacent normal tissue and from necrotic material. The concentration of tocopherol in the tumor tissue was then determined by a variation of the Emmerie-Engel method (27, 37, 40). Analyses were also made of liver from tumor-bearing rats and in some cases of other tissues as well. A 5–10-gm. sample was homogenized in 95 per cent ethanol and the lipid extracted for at least

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[1] Supplied by Dr. G. A. LePage of the McArdle Memorial Laboratory of this university.

18 hours with hot ethanol in a Soxhlet apparatus. The extracts were then refluxed for ½ hour with 200 mg. of KOH per flask, and extracted into petroleum ether, transferred to benzene, and chromatographed on Floridin[2] previously treated with $SnCl_2$ (37, 40). An aliquot of the eluate was transferred to absolute ethanol and treated with the Emmerie-Engel reagents in the Evelyn colorimeter, and the optical density (L value) corrected for the reagent blank, multiplied by the constant $171_{515\ m\mu,\ 15\ sec.}$ (μg of tocopherol/tube) and the appropriate dilution factors.

The $SbCl_3$ test was used to determine vitamin A in the unchromatographed extracts of certain hepatomas and the corresponding livers as a measure of contaminating liver tissue in the tumor; tumors due to aminoazotoluene are alleged to be free from vitamin A (13).

For the production of liver tumors, comparable groups of 12–15 young, adult male rats of the Sprague-Dawley[3] or Holtzman strain,[4] weighing approximately 200 gm., were fed 3′-Me-DAB in three types of experiments: the intake of vitamin E was varied while the dye was fed, after the feeding of the dye, or during a period of interruption in the administration of the dye. Weights were recorded at intervals, and in addition to the diets each rat also received 2 drops of halibut liver oil every 4 weeks. Basal diet I contained 5 per cent of corn oil, while a diet relatively low in vitamin E (diet III) contained 4 per cent of lard and 1 per cent of corn oil. In the series in which the administration of the dye was interrupted, the rats were fed several modifications of the basal diet during the intermediate period (Table 4).

The carcinogen 3′-Me-DAB was incorporated into the ration by evaporating an ether solution of the dye onto the dry ingredients. The fat with or without the additional tocopherol was then incorporated into the diet and the mixture passed through a 10-mesh screen. In an attempt to determine a relationship between vitamin E and factors previously associated with the carcinogenicity of the azo dyes, the concentration of riboflavin in the liver was determined fluorometrically (1, 7), and measurements were made of the coagulability of liver protein (14) and of the destruction of DAB by liver slices *in vitro* (24). The rats for these experiments were fed either diet I or diet II or similar rations containing 0.064 per cent of 3′-Me-DAB for 4 weeks.

[2] Floridin Co., Warren, Pa.

[3] Sprague-Dawley Co., Madison, Wis.

[4] Holtzman-Rolfsmeyer Co., Madison, Wis.

RESULTS

Accumulation and retention of tocopherol by tumor tissue.—When rats bearing hepatomas were fed a diet high in α-tocopherol, there was a rapid uptake of the vitamin by the tumors as well as by the livers. The average tocopherol content of the livers from the low tocopherol group was 34.6 μg/gm and that of tumors 9.2 μg/gm, as compared to 81.4 μg/gm and 29.0 μg/gm, respectively, in livers and hepatomas of rats on diets supplemented with α-tocopherol (diet II) for 4–10 days (Table 1, Groups 1 and 2). When at least 10 days elapsed before analysis, the livers and tumors contained 111 and 64.1 μg of tocopherol/gm, respectively (Table 1, Group 3). The latter figures indicate a 7-fold increase in the tocopherol content of the tumor, while the increase in the liver was only 3.3-fold.

When a group of tumor-bearing rats which had been on the high tocopherol diet (II) for 8 days was fed basal diet I for another 8 days, the livers contained 56.7 μg of tocopherol/gm, a loss of approximately 50 per cent, while the tumors still held 47.7 μg/gm, a loss of only 25 per cent (Table 1, Groups 3, 4). In a second experiment, the vitamin A content of both tumor and liver was determined along with tocopherol. The amounts of tocopherol in the tissues before and after supplementation were similar to those in the previous series (Table 1, Group 5 versus 1 and 3), and after "depletion" on diet I for 10 days, the average tocopherol content of livers and tumors fell to 70.4 and 32.2 μg/gm, respectively (Table 1, Group 6). This decrease in tumor tocopherol, however, was regarded as more apparent than real, for during the 20 days of the experiment the tumors had increased in size 2–10-fold. The vitamin A content of two livers and tumors from this group was 49.8 μg/gm and 9.0 μg/gm, respectively. If pure hepatoma due to 3′-Me-DAB is devoid of vitamin A (13), then the tumor material examined was "contaminated" by 10–40 per cent of normal liver.[5] Even with this correction, however, the tumors still displayed a marked ability to take up and to hold vitamin E.

The other tumors studied proved to be like liver tumors in their ability to accumulate tocopherol. In the first experiment the tocopherol content of the Flexner-Jobling carcinoma rose from 8.2 μg/gm on the low tocopherol diet to 38.9 μg/gm on the high tocopherol diet; in the second it rose from 4.6 to 10.6 μg/gm. In both experiments the absolute concentrations of tocopherol were less in the

[5] Another possibility is that the vitamin A in the tumor is part of a residue left by vanished liver cells that had been trapped by the tumor.

tumor than in the liver, but the relative increases on supplementation were greater for the tumor than for liver (Table 2). Both the Jensen sarcoma and the Walker carcinoma resembled the other tumors in their ability to accumulate vitamin E. Contamination with normal tissue was possible for the Jensen sarcoma, since it grew intramuscularly, but normal muscle contained considerably less tocopherol/gm than the tumor.

Rats bearing hepatomas were available from an experiment in which the administration of azo dye was interrupted and the tocopherol intake varied for 4 weeks during the intermediate period (tumor results in Table 4). This difference in diet did not affect the tocopherol content of the liver 13 weeks later (27.6 versus 28.5 μg/gm, Table 1, Groups 7 and 8), but the tocopherol content of the tumors varied with previous dietary history, 6.8 versus 11.4 μg/gm. Since tumors were not evident at the time the diet had been varied, the normal tissues of the rat must still have contained "excess" tocopherol at the time the tumors began to grow.

Experiments on tumor formation.—The level of tocopherol fed with 3′-Me-DAB did not appear to have any significant effect on the percentage of liver tumors that developed (Table 3, Groups 13–18), nor were any consistent differences noted in food intake, survival, weight, or in the development of cirrhosis or of white necrotic areas. When a diet high in tocopherol was fed only after the administration of the carcinogen, tumor incidence was diminished somewhat (Table 3, Groups 19

TABLE 1

THE TOCOPHEROL AND VITAMIN A CONTENT OF HEPATOMAS AND LIVERS OF RATS FED VARIOUS DIETS

Group	Diet	No. of rats	Conc. of tocopherol in Liver (μg/gm)	Conc. of tocopherol in Hepatoma (μg/gm)	Conc. of vitamin A in Liver (μg/gm)	Conc. of vitamin A in Hepatoma (μg/gm)
1	Diet I*	3	34.6± 5.9†	9.2± 1.8†		
2	Diet II,‡ 4–10 days	3	81.4±11.4	29.0± 6.3		
3	Diet II at least 10 days	3	111 ±26	64.1±13.7		
4	Diet II, 8 days, followed by diet I, 8 days	4	56.7± 8.2	47.7± 3.5		
5	Diet I	1	37.2	4.9	52.0	9.1
5	Diet II, 10 days	1	125	74.8	42.0	16.8
6	Diet II, 10 days, followed by diet I, 10 days	5	70.4±11.4	32.2±11.8	49.8(2)	9.0(2)
7	Diet II, 4 weeks, followed by diet III, 13–17 weeks§	8	27.6± 9.4	11.4± 3.8		
8	Diet III, 17–21 weeks‖	9	28.5± 5.1	6.8± 2.8		

* The tocopherol content of diet I was estimated as 6.3 μg of α-tocopherol and 44.7 μg of γ-tocopherol/gm.

† Standard deviation.

‡ Diet II contained an additional 1 mg of α-tocopherol/gm in Groups 5–7 and 1.6 mg/gm in Groups 2–4.

§ Diet II, 4 weeks; diet III plus 0.064 per cent of 3′-Me-DAB, 5 weeks; diet III, 8–12 weeks. Diet III contained 4 per cent of lard and 1 per cent of corn oil.

‖ Diet III, 4 weeks; diet III plus 0.064 per cent of 3′-Me-DAB, 5 weeks; diet III, 8–12 weeks.

TABLE 2

THE ACCUMULATION OF TOCOPHEROL BY CERTAIN TUMORS AND NORMAL TISSUES OF RATS

Group	Tissue	No. of rats	Tocopherol content: Low toc. Diet I* (μg/gm)	Tocopherol content: High toc. Diet II† (μg/gm)	Per cent increase
9	Flexner-Jobling carcinoma	2	8.2± 0.4‡	38.9± 7.2‡	370
9	Liver	2	36.7± 2.4	83.2± 3.3	127
9	Muscle	2	8.6± 0.5	21.0± 0.2	144
10	Flexner-Jobling carcinoma	4	4.6± 1.3	10.6± 3.6	130
11	Jensen sarcoma	3	10.6± 4.6	27.8± 7.3	162
12	Walker carcinoma	5	3.0± 1.8	17.4±10.0	480
10–12	Liver	3	40.2±11.9	45.5±11.0	13
10–12	Muscle	3	4.4± 3.0	8.0± 6.0	82

* The tocopherol content of diet I was estimated as 6.3 μg of α-tocopherol and 44.7 μg of γ-tocopherol/gm.

† In Group 9, diet II contained an additional 1.6 mg of α-tocopherol/gm and was fed for 20 days. In Groups 10–12 1.0 mg of α-tocopherol/gm of diet was added and fed 11–16 days.

‡ Standard deviation.

and 20). The incidence of tumors was also relatively low when the dietary fat consisted mainly of lard rather than of corn oil (Group 21), paralleling an older observation (19) that lard delayed the development of liver tumors due to DAB. This latter effect presumably involves lipoidal substances other than tocopherol.

When the level of dietary tocopherol was altered at an intermediate period between two periods during which the carcinogen was fed (6), tocopherol appeared to enhance tumor formation slightly in three separate series (Table 4, Groups 22–23, 24–25, 28–29). The significance of this increase, however, was doubtful, since the replacement of the casein in diet I by 18 per cent of brewer's yeast also increased tumor incidence somewhat (Groups 26 and 30), and this latter dietary change presumably increases the need for vitamin E (15, 18). The presence of highly unsaturated fatty acids in the diet, 5 per cent linseed oil plus 5 per cent cod liver oil, resulted in irregular variations in tumor incidence (Groups 27 and 32).

The feeding of tocopherol did not alter the concentration of riboflavin in the liver, nor did it prevent the loss of riboflavin from the livers of rats fed 3′-Me-DAB (Table 5); nor did tocopherol alter the ability of liver slices to destroy DAB *in vitro*. The only positive effect of tocopherol appeared to be on the heat-stability of liver homogenates from rats fed 3′-Me-DAB; a high tocopherol

TABLE 3

THE EFFECT OF VITAMIN E ON THE DEVELOPMENT OF LIVER TUMORS DUE TO 3′-ME-DAB*

Group	Per cent of 3′-Me-DAB and time fed	Diet† during dye feeding	Diet† after dye feeding	Av. initial wt. (gm.)	Av. wt. end of dye-feeding period (gm.)	Survival at end of dye-feeding	Nontumor 16 weeks after dye-feeding: Cirrhosis or spots	Nontumor 16 weeks after dye-feeding: Nearly normal	No. of tumors 16 wks. after dye-feeding	Per cent tumors
13	0.064	I	I	232	236	11/13	3	3	5	45
14	for 7	II	II	232	252	11/13	3	3	4	36
15	weeks	III	III	230	229	12/13	2	5	5	42
16	0.040	I	I	232	275	14/15		2	12	86
17	for 12	II	I	233	262	13/15		3	9	69
18	weeks	III	I	230	271	15/15		4	11	73
19	0.056	I	I	211	196	11/11	1	2	8	73
20	for 8	I	II	209	197	12/13	2	6	4	33
21	weeks	I	III	221	195	12/13	2	6	4	33

* 3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene.

† Diet I contained 5 per cent of corn oil; diet II contained 5 per cent of corn oil plus 1.6 mg of α-tocopherol/gm in Groups 14 and 17 and 1 mg of α-tocopherol/gm in Group 20; diet III contained 4 per cent of lard and 1 per cent of corn oil.

TABLE 4

THE EFFECT OF DIET DURING THE PERIOD OF INTERRUPTION ON THE OCCURRENCE OF 3′-ME-DAB-INDUCED HEPATOMAS

(0.064 per cent 3′-Me-DAB, 4 wks.; dye-free diet, 4 wks.; 0.064 per cent 3′-Me-DAB, 4–5 wks.)*

Group	Diet during period of interruption	Av. weight (gm.) 0	4	8	12–13	Survival at 12–13 wks.	Nontumor at 21 wks.: Cirrhosis or spots	Nontumor at 21 wks.: Nearly normal	No. of tumors at 21 wks.	Per cent tumors
22	Basal diet I	204	191	261	252	7/7	0	3	4	57
23	Diet II: 1.3 mg of α-toc/gm	191	198	257	249	7/7	2	0	5	71
24	Basal diet I	236	192	259	230	10/10	3	1	4	40
25	Diet II: 1.3 mg of α-toc/gm	246	204	292	269	15/15	1	4	8	53
26	18 per cent brewer's yeast	241	203	265	224	15/15	4	1	9	60
27	5 per cent linseed oil, 5 per cent cod liver oil	238	202	274	253	12/15	3	5	3	25
28	Diet III: 4 per cent lard	216	180	279	262	15/15	1	4	10	67
29	Diet II: 1 mg of α-toc/gm	218	182	263	249	15/15	1	1	12	80
30	18 per cent brewer's yeast	212	183	264	245	14/15		0	14	100
31	18 per cent brewer's yeast plus 0.33 mg of α-toc/gm	214	184	267	250	14/15		0	13	93
32	5 per cent linseed oil, 5 per cent cod liver oil	214	180	265	255	12/15		0	12	100

* Four weeks in Groups 22–27; 5 weeks in Groups 28–32; Groups 22–23, data of W. L. Miller, Jr.; Groups 24–27, data of H. W. Rumsfeld, Jr.

diet retarded the coagulation of such homogenates (Table 5). In the absence of azo dye, however, tocopherol did not affect coagulability.

DISCUSSION

The present results suggest that α-tocopherol differs from the other fat-soluble vitamins in that tumor cells absorb it at least as rapidly as normal tissues and lose it more slowly when the animal is subsequently fed a diet low in vitamin E. The tocopherol in tumors appears to be more like vitamin C (30) or most members of the B complex (26) in that the concentrations in tumors are not very different from those in average normal tissues but less than those in liver. Vitamin C disappeared somewhat more slowly from tumors than

TABLE 5

THE EFFECT OF VITAMIN E ON CERTAIN PROPERTIES OF RAT LIVER

	Dye-free diets		0.064 per cent 3′-Me-DAB	
	Low toc.*	High toc.†	Low toc.*	High toc.†
Liver riboflavin, μg/gm	21.6	20.2	16.0	17.2
Per cent DAB destroyed, *in vitro*‡	25	23		
Coagulation of liver homogenates by heat: time in minutes	1, 1, 2, 2	1, 1, 3, 3	2, 3, 5, 5, 20, >30, 30	9, >30, >30, >30, >30

* Diet I contained 5 per cent of corn oil.

† Diet II contained 5 per cent of corn oil plus 1.6 mg of α-tocopherol/gm.

‡ 100 μg. DAB incubated with 200 mg. liver slices for 30 minutes.

from the liver during the depletion of guinea pigs and accumulated more slowly during subsequent supplementation; however, neither the liver nor the tumor in the rat was affected by dietary ascorbic acid (41). Riboflavin also disappeared from tumors as rapidly as from the livers of mice placed on deficient diets (25). Another analogy to the accumulation of tocopherol by tumors is that of certain labeled amino acids which are incorporated into tumor protein at least as rapidly as into normal protein (2, 28, 35, 42, 44). On depletion certain of the labeled amino acids, like tocopherol, disappeared more rapidly from the liver than from the tumor (35).

Materials which localize selectively in tumors have been sought for many years. Dyes are known which stain tumors diffusely and inhibit tumor development, but which also stain the testes and lymph nodes and cause damage to liver and thymus (20, 21, 29). As one might expect, the quantitative distribution between tumor and normal tissues varies with the compound administered. Trypan blue tagged with I^{131} was found in higher concentration in liver, spleen, and kidney than in carcinoma (36). The iodine of N-iodoacetyl-I^{131} tryptophan, leucine, or phenylalanine appeared in higher concentration in mouse Sarcoma 37 than in the liver of the host, and untagged derivatives inhibited the growth of this tumor (11). Mammary tumors in chicken eggs accumulated P^{32} more rapidly than any other tissue except the bone of the developing embryo (12). Vitamin E can be regarded as one of this latter group of compounds. The possibility exists that tocopherol could be employed to transport a radioactive atom as a diagnostic tool or possibly even a chemotherapeutic agent. Fortunately, excess tocopherol disappears rapidly from liver when supplementation ceases.

SUMMARY

Hepatomas induced by 3′-Me-DAB accumulated dietary α-tocopherol more rapidly than did adjacent liver tissue; in depletion experiments hepatomas held tocopherol more tenaciously than normal tissue. The Jensen sarcoma, the Walker carcinoma, and the Flexner-Jobling carcinoma also absorbed tocopherol more rapidly than liver or muscle.

Dietary vitamin E decreased the incidence of hepatomas when large amounts of tocopherol were fed after the administration of 3′-Me-DAB. The vitamin increased tumor incidence slightly when fed between two periods of dye-feeding, while variations in the vitamin E intake made while the carcinogen was fed had little effect on final tumor incidence. Yeast protein fed during the intermediate period increased tumor incidence slightly whether the diet was high or low in tocopherol.

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Differences in Adrenal Responsiveness to Post-castrational Alteration as Evidenced by Transplanted Adrenal Tissue*

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INTRODUCTION

In inbred strains of mice the removal of the gonads is followed by one of three different types of histological response on the part of the adrenal cortex. In most strains, some months after gonadectomy typical hyperplastic changes in the adrenal cortex develop, and these altered cortices may then produce significant amounts of sex steroids (4, 5, 6, 12, 14). In some animals of these strains, this hyperplastic process will progress until a definite adenoma or even a carcinoma is formed. In the CE strain, however, rather soon after the areas of cortical hyperplasia appear, areas of carcinoma develop within them so that in essentially all these animals sex steroid-producing adrenal cortical carcinoma are well established within about 9 months after the animals have been castrated (15–19). In the line of A mice maintained in this laboratory, on the other hand, the morphology of the adrenal is altered but little after the animals are gonadectomized (12). Occasionally, small areas of cortical hyperplasia may develop, but we have seen no evidence of sex hormone production by these rather infrequently occurring small areas of hyperplasia. From a rather extensive study of the post-castrational changes that occur in F_1 hybrid mice resulting from the mating of different inbred strains of mice (9), it appeared that the type of adrenal alteration noted was inherited equally through male and female parents and that the tendency for the adrenals to become carcinomatous was dominant over that for hyperplasia to develop, but that both of these were dominant over the tendency for no marked cortical alteration to appear. Since no F_2 or back-cross generations were studied, no idea of the number of genetic factors involved in this inheritance was obtained.

The purpose of the present study was to determine, if possible, whether these differences in adrenal response are due primarily to differences in the responsiveness of the genetically different end organs (adrenals), or primarily to differences in the alterations of the endocrine system produced by castration in the various strains of mice. In other words, is the lack of significant post-castrational adrenal alteration in A mice, for instance, the result of a nonresponsive end organ or is it due to a quantitatively and/or qualitatively inadequate change in the endocrine system of this strain of mice so that even a responsive adrenal cortex is not altered? It would seem that an answer to this question might be obtained if the cortical tissues of two different strains of mice were placed in the same environment and thus subjected to the "external" stimuli.

Since the maintenance of transplants of both normal and malignant tissue is dependent upon genetic factors (1, 2, 11), it is possible to transplant tissue from individuals of either parent inbred strain into F_1 hybrid animals resulting from the mating of the two inbred strains. Thus, it should be possible to place adrenal tissue of two inbred strains of mice into the environment of castrated, adrenalectomized F_1 hybrid animals and to observe the response of these genetically different adrenal tissues to the same stimulating factors. In such preparations, if the histological appearance of the transplanted adrenals simulated that seen in castrate animals of the donor strain, it would appear that the responsiveness of the end organ itself was the principal factor in determining the type of adrenal response noted following castration; while, if it simulated that seen in castrates of the recipient hybrid stock, differences in the ani-

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mal's response to castration would appear to be of the greater importance.

MATERIALS AND METHODS

The various inbred strains and hybrid crosses of the mice employed in this study are listed in Table 1. For convenience of reference, the type of post-castrational adrenal alteration noted in the various groups is also listed, as is the general type of sex steroid produced by these altered adrenals.

At the time of weaning, when both donor and recipient mice were from 4 to 6 weeks of age, the adrenals were removed from the donor animals, the periadrenal fat was dissected away, and the glands were cut in two. These four half adrenals were then implanted high in the axillary tissue of a recipient animal that was then immediately gonadectomized and adrenalectomized. Although it has been well demonstrated that the adrenal cortex will regenerate from the glomerulosa immediately under the capsule (10, 13), it was found in preliminary studies that a greater number of successful "takes" occurred when the adrenals were split open than when intact adrenals were grafted. For the first 2 or 3 months following the the surgical procedure the animals were maintained on a semi-synthetic diet in which the potassium salts of the salt mixture had been replaced by the corresponding sodium salts.[1] The animals also received 0.85 per cent sodium chloride and 3 per cent dextrose in their drinking water. It had been found previously that young adrenalectomized mice will grow and gain weight well and that female mice will show rather normal estrous cycles when maintained on this regimen. When sufficient time had elapsed to allow the adrenal grafts to regenerate, all animals were placed on a Fox Chow diet and given tap water to drink.

Beginning about 4 months after surgery, vaginal smears were taken monthly on all female animals. Animals were sacrificed when palpable adrenal transplants were noted, when their vaginal mucosae had shown evidence of estrogenic stimulation for several months, or when the animals reached 18 months of age. No animal dying before 1 year of age was included in the final tabulations. At autopsy a careful search for "extra-adrenal" cortical tissue (adrenal rests) was made employing a dissecting microscope, and the adrenal grafts, genital tracts, salivary glands, and mammary glands were removed for histological study. Serial sections of the entire adrenal graft were prepared for study except in those cases where a large adrenal carcinoma was found. Serial sections of many of the adrenal rests were also prepared for examination. In only seven cases were animals that were found dead in the cage and were too autolyzed for careful histological study included in the tabulation of results. These animals were included on the basis of the vaginal smear data obtained, since these data were found to correlate well with the autopsy findings as far as evidence of stimulation of the secondary sex organs was concerned.

TABLE 1

INBRED STRAINS AND HYBRID CROSSES EMPLOYED

Stock	A	Z	CE	AZF_1	$ACEF_1$	$ZCEF_1$
Histology of post-castrational adrenal change	Very minimal hyperplasia	Hyperplasia	Carcinoma	Hyperplasia	Carcinoma	Carcinoma
Types of sex hormones produced by altered adrenals	None	Feminizing	Feminizing and masculinizing	Feminizing	Feminizing and masculinizing	Feminizing and masculinizing

RESULTS

Since it has been shown that caloric restriction will somewhat delay the appearance of post-castrational adrenal cortical hyperplasia and will inhibit the production of sex steroids by histologically hyperplastic adrenals (3), the general physical status of the animals included in this study is of considerable importance. In preliminary experiments carried out to determine the best methods of transplantation, etc., inbred Z animals were used. It was found that, although the mortality rate was not excessive, the animals were generally not in good health and that, although hyperplastic changes occurred regularly in the grafted adrenals, the sex steroid output by these hyperplastic grafts appeared to be low as judged by the histological appearance of the secondary sex organs. When hybrid animals were employed, however, the mortality rate in the immediate postoperative period and again after the animals were transferred to the Fox Chow diet was low (the combined mortality was about 15 per cent), and the general health of the animals was almost uniformly excellent. This is attested by the fact that in the animals included in this report, no example of well developed adrenal alteration was found in an animal in which good evidence of sex steroid produc-

[1] This diet was kindly supplied by Dr. J. T. King.

tion was not also present. In two groups of animals, AZF_1 females bearing AZF_1 female adrenal grafts and AZF_1 females transplanted with Z male adrenals, the general health of many of the animals appeared somewhat below normal, however, and in these groups the degree of stimulation of the secondary sex organs was somewhat less than would be expected on the basis of the degree of histological alteration noted in the adrenal grafts.

Studies carried out in several inbred strains of mice and their F_1 hybrids had indicated that the latent period between castration and the appearance of a partially cornified vaginal epithelium as indicated by vaginal smears varied from strain to strain but was rather constant in individuals of a given strain or F_1 hybrid cross (9). It had originally been hoped that this aspect of the problem could also be studied in these animals bearing grafted adrenals. The vaginal smear data accumulated, however, indicated that in general the period between operation and the appearance of a sub-estrous smear is greatly prolonged in the case of the grafted adrenals and sufficiently variable as to preclude meaningful analysis. Whereas sub-estrous vaginal smears appeared in animals of the hybrid crosses studied here in from 4 to 8 months after castration, in the animals bearing adrenal grafts, relatively few showed sub-estrous smears before 10 months following operation, and many did not exhibit vaginal evidence of estrogen stimulation until from 14 to 16 months after operation.

A rather careful study was made of the extra-adrenal cortical tissue, since, if these deposits underwent hyperplastic or carcinomatous changes and produced significant amounts of sex steroids, interpretation of the function of the grafted adrenals would be made more difficult. Of the 128 animals autopsied (100 females and 28 males) and included in this study, adrenal rests were identified with the aid of a disecting microscope in 53 (in 42 females and 11 males). These usually occurred about the suprarenal vein, between it and the renal vessels, or on the inferior vena cava, but occasionally they were also found near the hilus of the kidney below the renal vessels. No rests were identified along the course of the mesiovarii or the ureters, but, owing to the large amount of abdominal fat, it was not possible to inspect these areas as closely as might be desired. In one animal which was excluded from the study, the suprarenal vein terminated in a small mass of hyperplastic cortical tissue, and it was assumed that this represented an instance of incomplete adrenalectomy rather than extra-adrenal cortical tissue. Multiple deposits of "rest" tissue were not infrequently encountered.

The rest tissue of 38 of the animals (44 individual rests) was sectioned serially for study. Histologically, these rests were found to be composed of rather normal appearing cortical cells which usually had no tendency to be arranged in a zonal pattern. Not infrequently a portion of the rest was composed of an eosinophilic staining hyaline material which appeared to be a residual of degenerated cortical cells. There was usually no definite adrenal capsule surrounding these nests of cortical cells. Only seven of the rests studied microscopically varied from the above pattern. Three showed an area with a definite capsule and a tendency for a zonal arrangement below the segment of capsule, and in two of these rests there was a definite proliferation of the small subcapsular cells. Two other rests had large areas of subcapsular proliferation with a fair number of large, foamy cells (Type B cells of Woolley) interspersed, which were suggestive of early cortical hyperplasia, and in one other rest a definite small nodule of typical cortical hyperplasia was found. The production of sex steroids by this latter rest could not be determined, since the animal also carried a grafted adrenal that showed extensive cortical hyperplasia. The seventh atypical rest had a medium-sized cluster of medullary cells to one side of the cortical tissue. Since the cortical tissue in this rest showed no tendency for a zonal arrangement and the medullary tissue lay to one side of the cortical tissue, it was felt that this represented extra-adrenal tissue rather than a remnant of an incompletely excised adrenal gland.

A summarization of the histological changes found in the adrenals grafted to female animals and the histological evidence of sex steroid production by these grafts is given in Tables 2–4. From an examination of these data, it is apparent that the histology of the adrenal grafts as well as the production of histologically detectable amounts of estrogenic hormones by them simulate rather well the situation seen in ovariectomized animals of the donor strains (Table 1). Of 27 pairs of A strain adrenals transplanted to AZF_1 or $ACEF_1$ hybrid females, only two became significantly hyperplastic and produced effective amounts of estrogen. This is in sharp contrast to the 42 grafts from Z, AZF_1, CE, and $ACEF_1$ donors transplanted to AZF_1 or $ACEF_1$ hybrid females, for 26 of these showed marked histological alterations accompanied by the production of significant amounts of estrogen. As mentioned earlier, the adrenals of oöphorectomized A strain female mice occasionally show some small areas of hyperplasia, but these apparently do not produce detectable amounts of sex steroids. It is of some interest, then, that in the

$ACEF_1$ female in which the grafted A adrenal produced significant amounts of estrogen, only one of the four adrenal fragments had undergone hyperplastic change, while the other three retained their normal histological pattern. Also in the AZF_1 animal in which the A adrenal graft had become hyperplastic, about one-half of the graft recovered at autopsy was composed of normal cortical tissue. By contrast, the usual picture seen in the grafted Z or AZF_1 adrenals was that of a much more extensive cortical hyperplasia with less residual normal cortex. This would suggest that even in the two instances of hyperplasia of grafted A strain adrenals the degree of anatomical alteration found was somewhat less than that seen in the hyperplastic grafts from Z or AZF_1 animals.

TABLE 2

AZF_1 FEMALE MICE BEARING ADRENAL TRANSPLANTS

Donor stock	No. animals	Genital tract	Histology of transplant
A	12	1 stimulated	1 hyperplastic
		11 atrophic	10 normal; 1 not autopsied
F_1	10	6 stimulated	6 hyperplastic
		4 atrophic	2 minimal hyperplasia; 2 normal
Z	14	12 stimulated	12 hyperplastic
		2 atrophic	1 minimal hyperplasia; 1 normal (small)
Z Male	11	7 stimulated	7 hyperplastic
		4 atrophic	2 minimal hyperplasia; 2 normal

TABLE 3

$ACEF_1$ FEMALE MICE BEARING ADRENAL TRANSPLANTS

Donor stock	No. animals	Genital tract	Histology of transplant
A	15	1 stimulated	1 hyperplastic
		14 atrophic	10 normal; 1 minimal hyperplasia; 3 not autopsied
F_1	12	6 stimulated	5 carcinoma; 1 hyperplastic
		6 atrophic	2 minimal hyperplasia; 4 normal
CE	6	2 stimulated	1 carcinoma; 1 hyperplastic
		4 atrophic	3 normal; 1 not autopsied

It should be emphasized that the hybrid animals bearing grafted A strain adrenals appeared to be in as good health as did those bearing the other types of grafts and that, in most instances, significant amounts of rather normal appearing cortical tissue were recovered at autopsy. The histology of the cortical tissue found in these adrenals, 12–17 months after transplantation, was not significantly different from that described by other authors in rats (10, 13). The variation from graft to graft was considerable. In some, large clumps of cortical cells were seen which appeared to be somewhat larger than normal and showed only a slight tendency to possess the typical zonal arrangement seen in *in situ* adrenals (Fig. 1). In other grafts, however, the architecture of the normal adrenal had been reconstructed rather well (Fig. 2). There were all variations between these two extremes in the grafts found in different animals and even between different fragments of the grafts in the same animal. There frequently was residual evidence of the initial degeneration that has been described as occurring immediately after transplantation (10). This usually consisted of areas of hyaline material which sometimes contained "crystal clefts" or, even rarely, areas of calcification. Although the majority of all the animals included in this study had grafts that contained goodly amounts of either normal or altered cortical tissue, a few of the animals that survived more than a year were found to have only very small amounts of residual adrenal graft tissue, but in most of these, the function of the small graft was apparently being augmented by the animal's own "rest" tissue.

TABLE 4

$ZCEF_1$ FEMALE MICE BEARING ADRENAL TRANSPLANTS

Donor stock	No. animals	Genital tract	Histology of transplant
Z	8	6 stimulated	5 hyperplastic; 1 not autopsied
		2 atrophic	1 minimal hyperplasia; 1 normal
F_1	8	6 stimulated	4 carcinoma; 2 hyperplastic
		2 atrophic	2 minimal hyperplasia
CE	6	4 stimulated	3 carcinoma; 1 not autopsied
		2 atrophic	1 minimal hyperplasia; 1 normal
Z and F_1	5	5 stimulated	F_1: 4 carcinoma; 1 hyperplastic Z: 4 hyperplastic; 1 scar

In the studies employing $ZCEF_1$ female recipients (Table 4) it is also evident that the histology of the post-castrational adrenal alteration was similar to that seen in the donor stock, for all five of the altered adrenals from donor Z animals showed only hyperplasia which was indistinguishable from that seen in the Z adrenals grafted to AZF_1 recipients (Figs. 4 and 5). On the other hand, in four of the six adrenals from F_1 donors and in all three of the adrenals from CE donors that showed significant post-castrational change, typical cortical carcinomas had developed (Fig. 6). It is probable that the sex steroids produced by the altered adrenal grafts materially affect the endocrine system of the recipient animals and thus alter the endocrine environment in which the grafts reside. It is also possible that the effect upon the host's endocrine system of grafted Z adrenals might differ from that of the grafted CE or $ZCEF_1$ adrenals and that such differences might be responsible for the development of carcinomas in

the CE and $ZCEF_1$ grafts, while the Z grafts remain hyperplastic. To preclude this possibility, a small number of adrenalectomized, ovariectomized $ZCEF_1$ females received a single Z adrenal graft in the right axilla and a single $ZCEF_1$ adrenal graft in the left. Since the two types of adrenal tissue were now in the same animal, it seems safe to assume that they were being subjected to the same stimuli throughout the entire experiment. Due to an unfortunate circumstance, only five such animals were available for final study. Although all the animals showed subestrous smears before they were a year of age, they were not autopsied until they were sixteen months of age in order to assure a complete development of the histological alteration in the adrenals. As can be seen in Table 4, four of the five F_1 hybrid adrenals were carcinomatous, while four of the five Z adrenals showed only cortical hyperplasia. It would appear, then, that the histological type of adrenal alteration noted after castration is also a reflection of the responsiveness of the adrenal tissue itself.

Although all the evidence obtained from grafting the adrenals of female mice would indicate that end organ responsiveness per se plays the major role in determining the type of adrenal response noted in various strains of mice after oöphorectomy, the situation in male mice remains of considerable interest. It had been found previously in certain strains of mice, e.g., Z strain, that, whereas oöphorectomy is routinely followed by extensive hyperplastic changes in the adrenals with the production of goodly amounts of estrogenic hormones, orchiectomy results in much less adrenal alteration and considerably less production of estrogen. In other stocks of animals, e.g., CE and $ZCEF_1$, on the other hand, both the degree of histological alteration noted and the production of sex steroids by these altered adrenals seem to be about the same in the two sexes. The question then arises whether this dissimilarity in the response of the adrenal in male and female Z animals is also due to differences in responsiveness of the end organ itself or to differences in the hormone alteration effected by gonadectomy in the two sexes.

Two experiments were set up to investigate this problem. In the first, a group of oöphorectomized, adrenalectomized AZF_1 female animals was grafted with Z male adrenals. As can be seen in Table 2, seven of eleven of these male adrenal grafts became extensively hyperplastic and produced detectable amounts of estrogen. As mentioned earlier, for some reason the general health of this group of animals was somewhat inferior to that usually seen in the animals reported here, and the degree of stimulation of the secondary sex organs was correspondingly less than that seen in the healthier AZF_1 females bearing Z female adrenals; but it was approximately the same as that seen in the AZF_1 female recipients bearing AZF_1 female grafts (this latter group of animals tended also to be in somewhat poorer general health). In the second experiment, Z male adrenals were grafted into castrated, adrenalectomized $ZCEF_1$ male mice, and the results of this experiment are summarized in Table 5. In nine of the fourteen animals bearing Z male adrenal grafts and living to 18 months of age, the grafts showed extensive hyperplastic changes which were indistinguishable from those seen in the Z female adrenals grafted to $ZCEF_1$ female mice. The mammary glands of these animals showed extensive mammary gland development which was equal to that seen in the $ZCEF_1$ male animals bearing $ZCEF_1$ male adrenal grafts. It would thus appear that the Z male adrenal gland itself is inherently capable of undergoing an extensive hyperplastic change and that in all probability the dissimilarity of adrenal response between gonadectomized Z male and female animals is caused by a difference in the hormonal alteration effected by gonadectomy—possibly a difference in the response of the hypophysis in the two sexes. It should be noted, however, that, although the degree of response of these Z male adrenals appears to have been significantly increased by placing them in the environment of castrated, adrenalectomized $ZCEF_1$ hybrid males, the histological type of this response remains characteristically that of Z mice, namely, hyperplasia, since none became carcinomatous as did some of the grafted $ZCEF_1$ male adrenals. The reason for the poor results obtained with grafted CE male adrenals is not immediately evident, but,

TABLE 5

$ZCEF_1$ MALE MICE BEARING TRANSPLANTED ADRENALS

Donor stock	No. animals	Mammary glands	Histology of adrenal transplant
Z	14	10 well developed	9 extensive hyperplasia*
		3 minimally stimulated	3 moderate hyperplasia
		1 no development	1 normal
F_1	7	5 well developed	3 carcinoma; 2 extensive hyperplasia
		1 minimally stimulated	1 moderate hyperplasia
		1 no development	1 small amount of hyperplasia
CE	7	1 minimally stimulated	1 normal
		6 no development	4 minimal hyperplasia; 2 normal (considerably hyalinized)

* In one of these animals the adrenal graft was lost during preparation for microscopic examination.

on histological examination, considerable hyalinization of these grafts was noted.

The only major difference noted in the response of grafted adrenals to that seen in *in situ* adrenals was in the production of significant amounts of masculinizing hormones. The adrenal carcinomas that arise in gonadectomized CE mice, both male and female, frequently produce significant amounts of masculinizing as well as feminizing hormones (15–19). This is also true of the adrenal carcinomas developing in $ACEF_1$ and $ZCEF_1$ hybrid mice. In none of the animals bearing grafted CE, $ACEF_1$, or $ZCEF_1$ adrenals was there evidence of any significant masculinization from the histological examination of their salivary glands, kidneys, or seminal vesicles. The reason for the failure of these carcinomas developing in grafted adrenals to produce detectable amounts of androgenic hormones is not evident at the present time.

It is of some interest to record the presence of normal-appearing medullary tissue in these adrenal grafts. Although the adrenals were not prepared so that stains for chromaffin could be applied, since our primary interest was in the cortical changes, the appearance of the medullary cells is sufficiently characteristic in the routine hematoxylin-eosin preparation to allow easy identification. In 93 of the 127 adrenal grafts studied, histologically normal-appearing medullary cells were found. These cells were arranged in cords or clumps of varying sizes, and not infrequently where the morphology of the graft had not been too extensively altered either by transplantation or by post-castrational changes the location of these cells was similar to that seen in the *in situ* adrenals (Figs. 2 and 3). Whether this medullary tissue was functioning could not, of course, be determined by histological examination. However, it is of considerable interest that in most of these transplanted adrenals histologically normal medullary tissue was in evidence 12–17 months after the tissue had been transplanted, since there appears to be some species variation in the matter of the maintenance of grafted medullary tissue (for review see [7]). It is apparent, however, that in mice the medullary tissue need not be removed prior to the transplantation of cortical tissue and that significant amounts of medullary tissue will be maintained if the adrenals to be grafted are cut through to allow earlier vascularization of the medullary zone. In addition to the normal medullary tissue, areas of basophilic hyaline material were also frequently encountered which appeared to have resulted from a degeneration of medullary tissue.

DISCUSSION

Many differences in the anatomy and function of the endocrine system have been described in intact and experimentally manipulated mice of various inbred strains. It seems very likely that many, if not most, of these differences are in some way controlled by genetic factors. In most instances it has been as yet impossible to determine to what extent such differences result from dissimilarities in the inherent reactiveness of the target tissues to similar degrees of stimulation or from differences in the general endocrine constitution of the different strains of mice resulting in differences in the stimulation to which the end organs are subjected. This problem can be studied in certain instances by placing the genetically dissimilar end organs from two inbred strains of mice into the identical environment of F_1 hybrid individuals arising from the mating of the two inbred stocks under consideration. In this way, genetically dissimilar tissue would be subjected to the same stimuli, and, in the case of grafted endocrine glands, the hormones produced by the genetically different glands would be metabolized in the same manner and act upon genetically similar secondary sexual tissues of the host animals.

In the initial experiments employing this technic, the histology and function of the ovaries of Z and A strain mice were compared (8). It was found that the differences in normal histology seen in the ovaries of these two strains of mice appeared to be due mainly to differences in the stimuli, probably pituitary in origin, that are acting upon the ovaries, for when these histologically different ovaries were placed in the environment of gonadectomized AZF_1 hybrid females they became histologically similar. More specifically, the A strain ovaries were altered histologically so that they became very similar in appearance to the ovaries of Z or AFZ_1 hybrid animals. The function of these histologically altered A strain ovaries appeared, however, to have certain characteristics that differed from those seen in the grafted Z strain ovaries suggesting that certain aspects of ovarian function were inherent in the grafted ovarian tissue itself.

The experiments reported here, in which the post-castrational alterations noted in grafted adrenals were studied, indicate rather clearly, however, that in this situation both the type of abnormal histological pattern that results and the function of this altered adrenal tissue are largely a reflection of a genetically controlled responsiveness of the adrenal tissue per se. In all instances, the type of post-castrational adrenal response noted in the grafted adrenals was that found in the adrenals of

gonadectomized animals of the donor stock. Adrenals from A strain mice when transplanted to AZF_1 or $ACEF_1$ recipients tended to show no particular post-castrational alteration and Z strain adrenals, whether grafted to AZF_1 or $ZCEF_1$ recipients, showed only hyperplastic changes with no increased tendency for carcinoma formation when the adrenals were in the environment of gonadectomized, adrenalectomized $ZCEF_1$ recipients. Although somewhat larger numbers of animals would have been desirable in each of the experimental groups, by pooling certain groups, numbers with greater statistical significance can be obtained. Thus, eighteen Z adrenals transplanted to $ZCEF_1$ recipients showed extensive post-castrational alteration, and in all instances serial sections of these grafts showed only hyperplastic changes, while of the nineteen CE or $ZCEF_1$ adrenal grafts showing extensive changes fourteen were carcinomatous. Some indication of variations in the response of the endocrine systems of different strains of mice to gonadectomy was, however, obtained. Thus, Z male adrenals, when transplanted to adrenalectomized, gonadectomized AZF_1 females or $ZCEF_1$ males, did show extensive hyperplastic changes and did produce effective amounts of estrogen. This finding would suggest that the dissimilarity in degree of adrenal hyperplasia noted in gonadectomized female and male Z strain animals results from a difference in the stimuli acting upon the cortical tissues of the two sexes following the removal of the gonads. Also, there were two instances in which grafted A strain adrenals did become more hyperplastic than is usually the case in oöphorectomized A strain mice, and in these instances detectable amounts of estrogen were produced. It would appear then, that although the lack of reactiveness of the adrenal tissue itself is primarily responsible for the lack of post-castrational adrenal alteration in A strain mice, the stimuli that these adrenals are subjected tc within gonadectomized A mice may also be somewhat less than those to which they are subjected when grafted to gonadectomized, adrenalectomized AZF_1 or $ACEF_1$ animals.

In general, the anatomic restoration and function of the adrenal tissue following transplantation appeared to be rather good throughout these experiments, and the subsequent development of the post-castrational alterations reproduced rather well that seen in *in situ* adrenals. A few differences, however, were evidenced. In the group of female mice studied, 69 received female adrenal grafts from strains of animals in which definite post-castration adrenal alterations occur. In 47, or 68 per cent of these, the adrenal grafts showed extensive histological alterations with estrogen production, whereas essentially 100 per cent of *in situ* adrenals in oöphorectomized females of these donor stocks showed the change. The time that elapsed between the grafting of the adrenal tissue and the appearance of a subestrous vaginal smear was usually considerably longer than the latent period between oöphorectomy and the appearance of vaginal subestrus in otherwise intact mice of the donor strains, and the variations from animal to animal in the length of this latent period were much greater in the case of the grafted adrenals. The "delaying" effect of transplantation is probably also reflected in the grafted CE, $ACEF_1$ and $ZCEF_1$ adrenals in that, of the 27 grafts showing definite post-castrational alteration, only twenty had become carcinomatous, whereas essentially all animals of the donor stocks would have cortical carcinomas by from 9 to 12 months after gonadectomy. Probably the only disturbing difference, however, was the failure of grafted CE, $ACEF_1$ and $ZCEF_1$ adrenals to produce significant amounts of masculinizing hormones.

The failure of development of cortical hyperplasia or cortical carcinoma in the majority of the adrenal rests of the recipient animals was very fortunate, for it made evaluation of the sex hormone production of the adrenal grafts much easier. That these deposits of cortical tissue do not generally undergo hyperplastic or carcinomatous changes in gonadectomized animals probably reflects their usual lack of a histologically distinct capsule and underlying glomerulosa in which these cortical changes appear to be initiated in the adrenal gland itself.

SUMMARY

By a series of experiments in which the adrenals of mice of inbred strains were transplanted into the common environment of adrenalectomized, gonadectomized F_1 hybrid animals, evidence has been obtained to indicate that the differences in the changes noted in the adrenals of different inbred strains of mice following gonadectomy are due mainly to differences in a responsiveness inherent in the adrenal tissue itself. When adrenals of A strain females (which show only minimal post-castrational changes) were transplanted to ovariectomized, adrenalectomized AZF_1 and $ACEF_1$ recipients, only 2 of 27 pairs became hyperplastic and produced detectable amounts of estrogen, whereas 26 of 42 grafts from donor Z, AZF_1, CE and $ACEF_1$ animals showed estrogen-producing hyperplastic or carcinomatous changes characteristic of the donor stock when transplanted to similar recipient animals. Similarly, Z adrenals, trans-

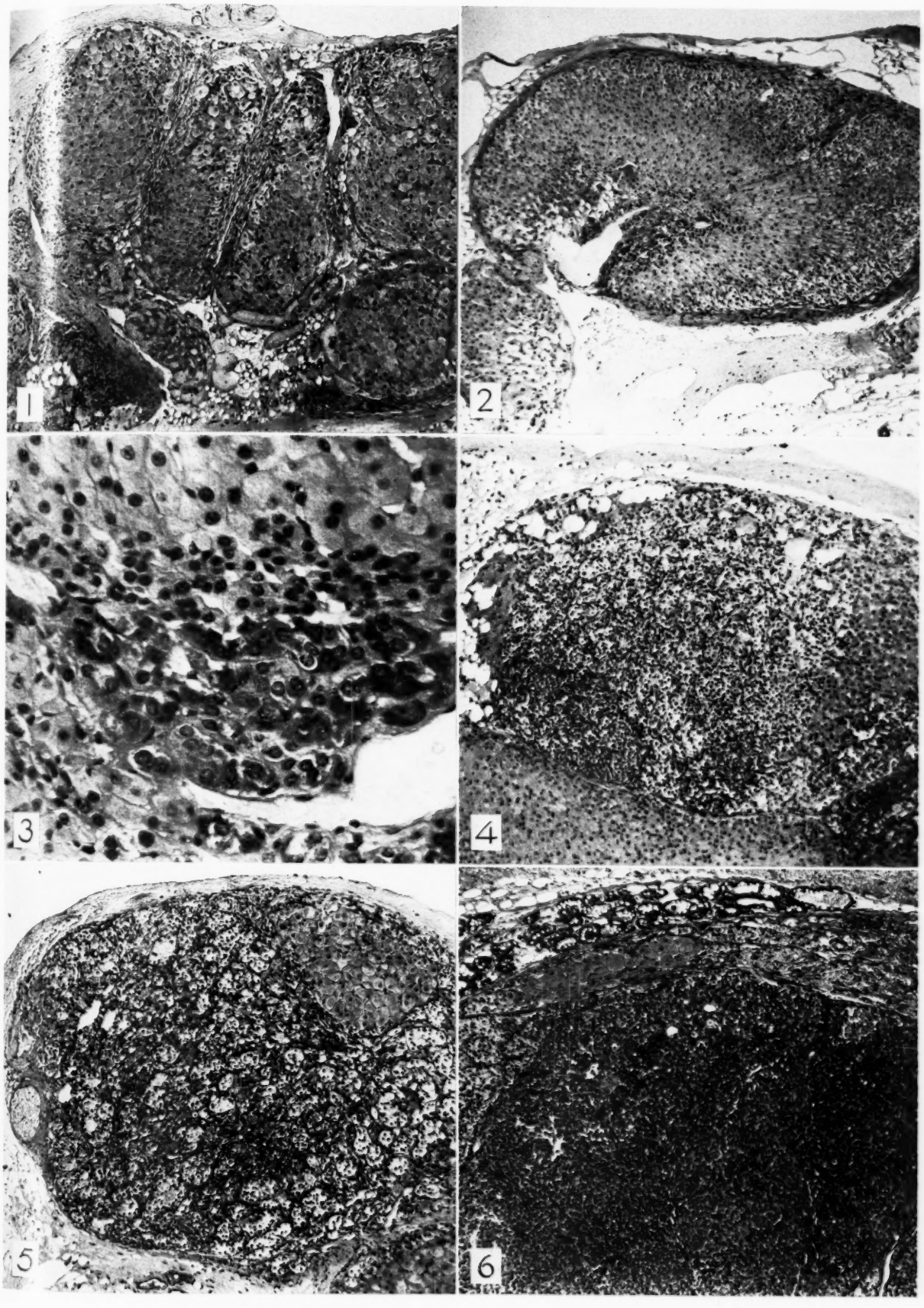
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FIG. 1.—An adrenal graft from an A strain donor that has been carried in an AZF_1 hybrid recipient for 17 months. The graft is composed of clumps of somewhat enlarged cortical cells with only minimal tendency for a zonal arrangement. Mag. ×70.

FIG. 2.—An adrenal graft from an A strain donor that has been carried in an AZF_1 hybrid recipient for 17 months. The histology of this graft rather faithfully reproduces that of an *in situ* adrenal. Note the definite zoning and the clump of medullary cells surrounding the central vein. Mag. ×70.

FIG. 3.—A high-powered view of a portion of the medullary tissue of the graft shown in Figure 2. Mag. ×350.

FIG. 4.—A portion of an adrenal graft from a Z strain donor that has been carried in an AZF_1 recipient for 17 months. Much of the graft is composed of hyperplastic cortex with some residual normal appearing cortex. Mag. ×70.

FIG. 5.—A portion of an adrenal graft from a Z strain donor that has been carried in a $ZCEF_1$ hybrid recipient for 16 months. The histology of the altered cortex is that of typical cortical hyperplasia with no evidence of carcinoma formation. Although the histology is somewhat different from that of the graft pictured in Figure 4, such minor degrees of variation were also present from graft to graft in recipients of the same hybrid cross so that no consistent difference was evident between Z strain grafts carried in AZF_1 and $ZCEF_1$ recipients. Mag. ×70.

FIG. 6.—A small portion of an adrenal graft from a $ZCEF_1$ donor carried in a $ZCEF_1$ recipient for 15 months. Most of the graft is composed of typical cortical carcinoma with a few areas of normal and hyperplastic cortex remaining. Mag. ×70.

planted to $ZCEF_1$ hybrid animals, showed only hyperplastic changes, while CE or $ZCEF_1$ adrenals, when transplanted to genetically identical recipients, tended to become carcinomatous. Evidence was obtained, however, to indicate that the lesser tendency for the adrenals of orchiectomized Z male mice to show extensive hyperplastic changes is caused, most probably, by a difference in the response of the endocrine system of Z male and female animals following gonadectomy.

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Response of the Central Nervous System of the Chicken to Methylcholanthrene: Failure To Induce a Neoplastic Process after 56 Months*

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INTRODUCTION

Tumors derived from nervous tissue have been induced with methylcholanthrene (3, 7), benzpyrene (8, 9), and dibenzanthracene (1) in mice and in rats when the carcinogen was used in a high concentration and when sufficient time was allowed for it to act. Methylcholanthrene has been the most successful of the three carcinogens employed when used in at least a 30 per cent concentration fused with cholesterol (5, 6). A dietary factor consisting of a periodic deficiency of thiamine and riboflavin was found to reduce significantly the length of the induction period in the methylcholanthrene tumors in rats (5, 6). Alteration of the intracellular metabolism of the cells from the riboflavin and thiamine deficiency was suggested as the factor causing the cells to respond more readily to the carcinogen. To study further the observed dietary effect on the induction period, it was decided to repeat the experiment with the use of another species. Chickens were selected, because intracranial tumors have not been previously induced in fowl, and their susceptibility to thiamine deficiency offered an opportunity to pursue further the reported effect of diet on the induction of intracranial tumors in rats.

The following communication reports these experiments on chickens, with the same experimental procedure and concentration of carcinogen that had proved successful in the production of glial tumors in rats and mice.

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EXPERIMENTAL PROCEDURE

Eighty-six pedigreed white leghorn pullets about 4 months of age and weighing approximately 700 gm. were employed.

Carcinogen.—Pellets of methylcholanthrene were made in a 30 per cent concentration prepared according to the technic of Peers (3). The methylcholanthrene was obtained from the Eastman Kodak Company. The technic of fusing the carcinogen with the chemically pure cholesterol has been described elsewhere (5, 6). When prepared, the pellets were 2 mm. in diameter, approximately 3 mm. in length, and weighed approximately 25.0 mg.

Operation.—The pellet was implanted in the right cerebral hemisphere without the use of anesthesia. The skin of the right superior aspect of the head was incised to the periosteum. A hole was made in the calvarium with a dental bur approximately 3 mm. in diameter. The pellet was inserted into the brain through the bur hole following an opening made in the dura with a pointed scalpel.

After insertion the pellet was pushed laterally so that it would not be extruded from the wound. The skin margins were approximated with a silk suture.

Dietary history.—Since it was planned to subject the chickens to periodic deficiency of thiamine, the danger of losing animals in the experimental periods would be lessened if mature animals were used. The periods of deficiency, therefore, were started following a 138-day growth period after the operation. During this time, the chickens were fed, ad libitum, Purina Broiler Chow, an all-sustaining ration for growing chickens. At the beginning of the experimental period there were 48 chickens surviving the operation and growth period. These chickens, averaging 1,625 gm., were divided into four groups of 12. Several chickens died of fowl leukosis and prolapsed intestine.

It was determined that the thiamine in the Purina Broiler Chow could be inactivated by autoclaving at 50 pounds pressure for 3 hours. Chemical analysis for thiamine, done through the courtesy of the Ralston Purina Company of St. Louis, for varying time periods was as follows:

Purina Broiler Chow (1 hr. autoclaving)—0.85 p.p.m.

Purina Broiler Chow (2 hr. autoclaving)—0.13 p.p.m.

Purina Broiler Chow (3 hr. autoclaving)—0.14 p.p.m.

It was felt that the reduced value of thiamine obtained in the 3-hour autoclaving would be sufficient to induce thiamine deficiency in the chickens. On this autoclaved food the chickens showed symptoms of thiamine deficiency characterized by leg, neck, and general body weakness beginning at about the fourteenth day. The chickens were subjected to three 25-day periods of deficiency in 6 months. Recovery periods of 30–40 days were allowed. In order to lengthen the period of deficiency with less risk of losing animals, thiamine hydrochloride was given intramuscularly to the experimental chickens when the deficient diet was discontinued. A dramatic clinical response was uniformly seen with the chickens, all resuming their normal eating habits. It was necessary to remove the chickens to a laboratory in another state where it was not possible to carry on the deficiency periods.

Necropsy and histologic technic.—A complete autopsy was performed on the chickens dying during the experimental period, and the six chickens that survived 4 years and 8 months after the implantation of the pellets. The calvarium was opened and the brain fixed in neutral 4 per cent formaldehyde (U.S.P. Formaldehyde 1:10). Following adequate fixation, the brain was sectioned with a razor blade, and a section 2 mm. in thickness was taken containing the pellet for microscopic study. Hematoxylin and eosin stains were employed.

RESULTS

Fifteen chickens survived for over 2 years; ten survived 3 years, and 6 were killed 4 years and 8 months after implantation of the pellet. None of the chickens dying during the experiment showed any clinical signs of intracranial tumor. No tumors were observed in the gross or microscopic examination of the brains. Study of the microscopic sections prepared from the brain at the site of the insertion of the pellet showed in all instances a broad zone of gliosis surrounding the pellet. Frequently, the area of gliosis contained small foci of calcification. The pellets were in contact with brain tissue and, usually, the overlying leptomeninges. Foci of macrophages, lymphocytes, and plasma cells were found in the glial scar and frequently in the subarachnoid space close to the inserted pellet. In those brains where the gliosis was prominent, the blocks were serially sectioned to be certain that the gliosis was not a neoplastic change.

COMMENT

The results of these experiments indicate that, under the conditions produced, the central nervous system of the chicken is resistant to carcinogenic stimulation by methylcholanthrene in a concentration that regularly produced tumors in mice and rats. Peers (3) reported glial tumors produced with pellets of methylcholanthrene in mice in 10 per cent concentration fused with cholesterol. The experiment was terminated at the end of 193 days, and 15 of the 32 tumors produced were of glial origin. Zimmerman and Arnold produced glial tumors in mice with pellets of pure methylcholanthrene (8, 9). The average exposure period for mice developing the 25 gliomas reported by these authors was 279 days. One of us (W. O. R.), using pellets of methylcholanthrene in 30 per cent concentration fused with cholesterol, as was used in the experiments reported here, induced fourteen tumors of glial origin in rats with an average exposure period of 299 days (5, 6). In those experiments, periods of riboflavin and thiamine deficiency were found to reduce significantly the induction period of the tumors (5, 6). It is not likely that the three periods of thiamine deficiency given the chicken in our experiments could be regarded as an adequate test to influence tumor development. The length of the experiment, however, is particularly significant.

The six chickens that lived 1,580 days following the implantation of the pellet represents $5\frac{1}{2}$ times the longest average exposure necessary for rats and mice to develop tumors. The fifteen chickens surviving for over 2 years were exposed for nearly 3 times as long as the highest average period necessary to produce tumors in rats and mice. It is unfortunate that the period of thiamine deficiency could not have been continued longer, since the altered metabolism of the brain due to the deficiency might have assisted the carcinogen in inducing tumors.

SUMMARY

Pellets of methylcholanthrene in 30 per cent concentrations fused with chemically pure cholesterol were implanted into the right cerebral hemisphere of 54 chickens. Thirty-one chickens survived for more than 2 years and six for 4 years

and 8 months. No tumors were induced. It may be concluded from these results that the central nervous system of the chicken is resistant to neoplastic change by carcinogenic stimulation of greater duration than will regularly produce it in rats and mice.

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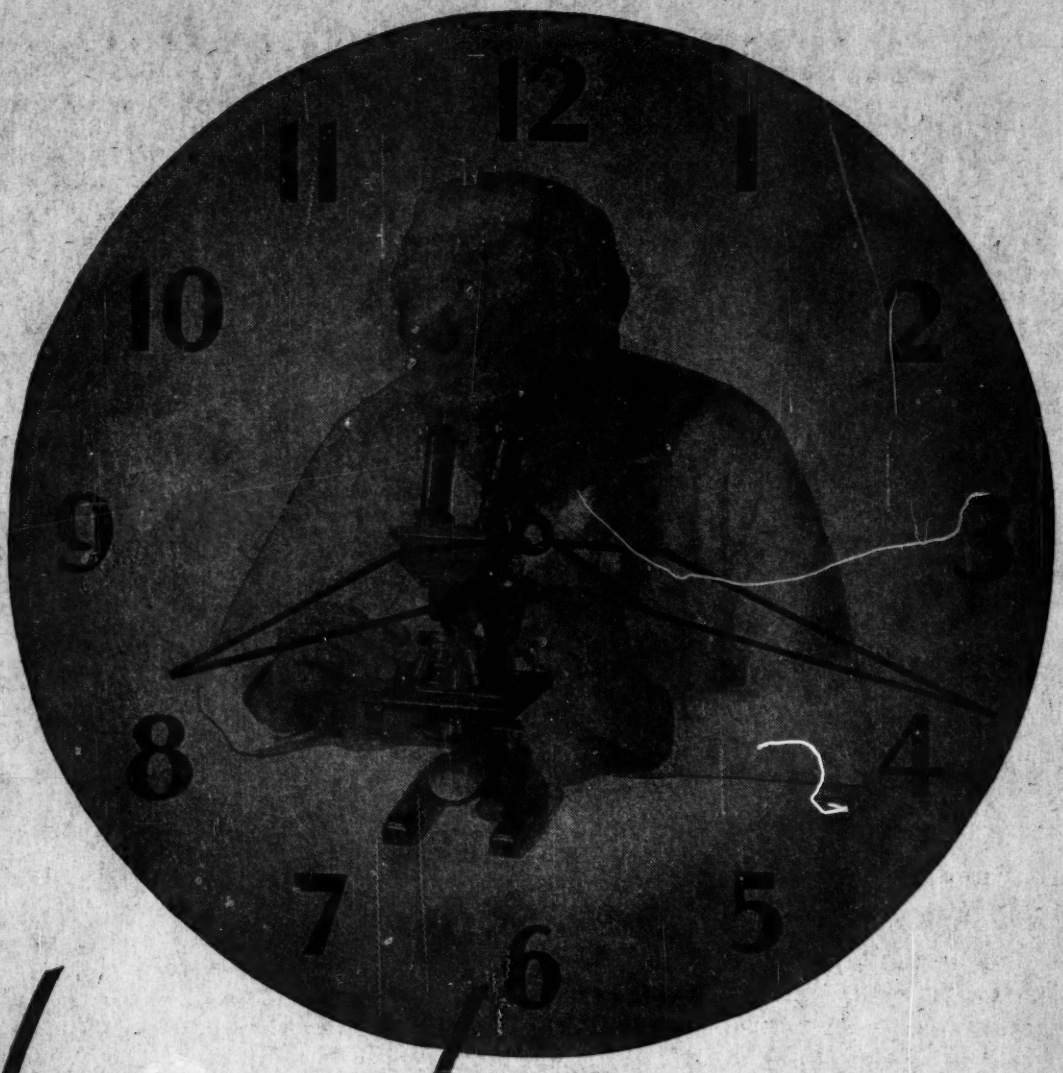

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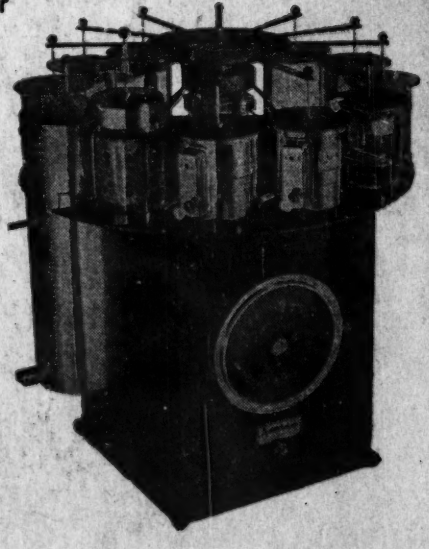

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